LANE MEDICAL LIBRARY STANFORD
G63 .C15 1925 OLD
Practical bacteriology, medical zoology
24503113433

LANE

MEDICAL LIBRARY

LEVI COOPER LANE FUND

Digitized by the Internet Archive
in 2025

https://archive.org/details/practicalbacteri0000vari_g1c9

PRACTICAL BACTERIOLOGY, MEDICAL ZOOLOGY AND IMMUNOLOGY AS APPLIED TO MEDICINE AND PUBLIC HEALTH

A GUIDE TO LABORATORY WORK

PRACTICAL BACTERIOLOGY MEDICAL ZOOLOGY AND IMMUNOLOGY

AS APPLIED TO MEDICINE AND PUBLIC HEALTH

A GUIDE TO LABORATORY WORK

PREPARED BY THE MEMBERS OF THE STAFF OF THE
UNIVERSITY OF CALIFORNIA MEDICAL SCHOOL
K. F. MEYER
Chairman

UNIVERSITY OF CALIFORNIA PRESS
BERKELEY, CALIFORNIA
1925

LANE LIBRARY

University of California Syllabus Series

No. 179. Issued September 15, 1925

Copyright, 1925
by the
Regents of the University of California

QR
185

PREFACE

The scope of bacteriological study has widened so considerably in recent years that the student finds some difficulty in utilizing textbooks for direct guidance in practical work. This condition has been appreciated by the Division of Bacteriology of the University of California, and in 1913 a syllabus setting forth in a comprehensive form the essential data and principles relating to pathological, bacteriological, and serological diagnosis was prepared. In accordance with the progress of the science of bacteriology, protozoology, and immunology, and owing to the fact that a separate department of pathology has been created, the original syllabus has undergone numerous revisions. Since the medical curriculum has been changed, fewer hours being assigned to bacteriology while additional time has been allotted to instruction in preventive medicine and medical zoology, it appeared desirable to prepare a new laboratory manual. The experiences of former years in teaching general, special, and diagnostic bacteriology and the organization of technician's training courses have suggested the arrangement of the subject matter. Inasmuch as the syllabus has been prepared for the use of students in the University of California Medical School the exercises and the special bacteriological and serological diagnostic methods are in accordance with our own system of teaching and the procedures practiced in the Clinical Laboratory of the University Hospital. Although primarily intended as a systematic guide to teach a student how to organize his work and to acquire in a short time the fundamental principles it is hoped that the syllabus will perpetuate interest in the science and serve as an aid in routine laboratory diagnoses during the clinical years. Emphasis is therefore placed in the daily exercises on the clinical and public health aspect of microbiology. The syllabus is in no way intended as an abbreviated textbook of medical bacteriology. In fact it represents a guide to the experimental methods, which always must supplement the purely didactic lectures on a biological science. The fundamental laws of infection and immunity are as far as possible taught by actual laboratory work. The student is assigned one or several problems, which are briefly outlined in a chapter of this manual. Without an adequate knowledge of the historical and theoretical facts it is impossible to comprehend the purpose of the experiment and to plan intelligently and soundly the intricate tests which are required to solve the problem. Comprehensive references are therefore included in the text. The student is expected to use them in his collateral reading.

Owing to the fact that the best methods or technical procedure employed in medical bacteriology are frequently published in periodicals or textbooks not available to the student it has been found necessary to incorporate in an appendix the preparation of stains, reagents, and culture media. The formulas have been tried out by members of the staff or by advanced students and have been found to give uniformly satisfactory results.

It is needless to emphasize that the writers of the manual are indebted to the authors of many works on bacteriology. Sections of the former syllabi have either been transferred to the present text or modified to suit the new requirements. Grateful acknowledgment is made to the authors of these

61092

sections. It is a source of great pleasure to acknowledge the assistance of Dr. Maurice C. Hall and Dr. V. Chapin, Zoologists of the Bureau of Animal Industry, U. S. Department of Agriculture, in supplying a complete list of the parasites found on laboratory animals, in giving the correct terminology, and in finding the scattered literature. Without the useful suggestions and the faithful assistance of my collaborators this manual could not have been completed.

K. F. Meyer,
Chairman.

CONTENTS

PAGE

Preface v

PART I. INTRODUCTION TO PRACTICAL BACTERIOLOGY, MEDICAL ZOOLOGY AND IMMUNOLOGY

Prepared by K. F. MEYER

I. General instructions 1
General laboratory rules 1
Special laboratory rules for safety 2
Rules of the culture media department 3
Supplies and apparatus 3
Reading references and their use 5
Preparation of papers and theses 10

II. General laboratory technic 11
Bacteriologic glassware 11
Care of instruments 16
Use of the microscope 17
Examination of cultures and specimens for the presence of micro-organisms 19
Cultivation of bacteria 23
Preparation of culture media 23
Technic of inoculation 31
Separation of bacterial mixtures and purification of cultures 33
Methods of anaerobic cultivation 38
Identification and classification of unknown organisms 40
Glossary of terms used on descriptive chart 49

III. Animal experimentation and autopsy notes on experimental animals 52
Spontaneous infections in laboratory animals 52
Rules regarding experimental animals 58
Animal inoculations 59
Observations of experimental infections during life 64
Animal autopsy 68
Histological examination of tissues 75

PART II. WEEKLY EXERCISES IN PRACTICAL BACTERIOLOGY

Prepared by P. SCHOENHOLZ

1st week

I. Preparation of glassware 83
II. Preparation of stains 83
III. Examination of young and old cultures of B. prodigiosus and Staphylococcus albus 83
IV. Examination of a pure culture of B. subtilis 84
V. Examination of organic infusion 84
VI. Qualitative examination of aerobic bacteria in the air 86

		PAGE
2d week		
VII.	Examination of yeasts	88
VIII.	Examination of molds	89
IX.	Study of pure cultures	90
3d week		
X.	Isolation and cultivation of bacteria	90
XI.	Preparation of culture media	92
XII.	Experiments in disinfection	93
4th week		
XIII.	Study of an anthrax infection	109
XIV.	A. Study of a pure culture of B. subtilis	112
	B. Study of aerobic spore-bearing bacilli related to B. anthracis	113
5th and 6th weeks		
XV.	Microscopic examination of infants' stools	116
XVI.	Microscopic examination of normal adults' stools	116
XVII.	Study of pure cultures of B. typhosus, B. paratyphosus A and B, and B. proteus	116
XVIII.	Isolation and identification of members of the colon-typhoid group from infected stool specimens	117
7th week		
XIX.	Widal reaction	121
XX.	Identification of organisms belonging to the group of dysentery bacilli	126
XXI.	Isolation of cholera vibrios from infected stools	127
XXII.	Water analysis	128
8th week		
XXIII.	Identification of the organisms belonging to the brucella group	133
XXIV.	Examination of members of the pasteurella group	134
XXV.	Study of B. pestis	135
XXVI.	Demonstration of animals infected with B. tularense	135
XXVII.	Examination of the organisms of swine erysipelas	136
XXVIII.	Bacteriologic examination of milk and ice cream	136
9th week		
XXIX.	Methods of staining tubercle bacilli in infected specimens	140
XXX.	Demonstration of tuberculous rabbits and guinea-pigs	141
XXXI.	Methods of isolating tubercle bacilli	141
XXXII.	Demonstration of tubercle bacilli by animal inoculation	142
XXXIII.	Demonstration of B. smegma, B. leprae and other acid-fast bacteria	143
XXXIV.	Laboratory diagnosis of C. diphtheriae	144
10th week		
XXXV.	Isolation and identification of organisms of the staphylococcus group	152
XXXVI.	Isolation and identification of organisms of the streptococcus group: Throat flora	153
11th week		
XXXVII.	Study of Diplococcus pneumoniae	155
XXXVIII.	Study of a pure culture of B. pneumoniae (Friedlander)	163

12th week PAGE

XXXIX. Study of a pure culture of Micrococcus intracellularis meningitidis ... 164
XL. Methods of detecting Meningococcus carriers ... 167
XLI. Identification of the microorganisms in specimens of spinal fluid ... 168
XLII. Study of hemoglobinophilic bacteria ... 169
XLIII. Methods of isolating B. influenzae ... 170

13th week

XLIV. Study of Diplococcus gonorrhoeae ... 172
XLV. Study of pathogenic branched bacteria ... 172
XLVI. Study of B. mallei ... 172
XLVII. Study of pathogenic fungi ... 173

14th week

XLVIII. Study of anaerobic sporulating bacteria ... 175

15th week

XLIX. Examination of smears containing fusiform bacilli ... 180
L. Study of spirochaetes ... 180
LI. Study of filterable viruses ... 181
LII. Study of Rickettsia bodies ... 182

PART III. PROBLEMS IN EXPERIMENTAL PATHOLOGY AND IMMUNOLOGY

2d week Prepared by B. G. Anderson

Experiment

1. Staining methods for distinguishing dead and living bacteria ... 187
2. Demonstration of optimum Ph of growth ... 187
3. Demonstration of involution forms ... 187
4. Demonstration of luminescent bacteria ... 188
5. Mechanism of the Gram stain ... 188

3d week

6. Bactericidal action of normal sera ... 189
7. One-cell culture by the Burri method ... 190
8. Mutation ... 190
9. Fever production ... 191
10. Inflammation ... 191

4th week

11. Preparation of a vaccine ... 192
12. Cuti-infection and cuti-immunity in anthrax ... 196

5th week

13. Pfeiffer's phenomena in vivo ... 197
14. Pfeiffer's phenomena in vitro ... 198
15. Effect of salt on agglutination ... 199
16. Acid agglutination ... 200
17. Protection experiment ... 201

6th week PAGE

A. Artificial antibody production—General References ... 202
18. Production of B. typhosus antiserum ... 207
19. Production of B. paratyphosus A antiserum ... 207
20. Production of B. paratyphosus B antiserum ... 207
21. Production of polyvalent B. typhosus, B. paratyphosus B. antiserum ... 207
22. Production of B. dysenteriae antiserum ... 207
23. Production of B. enteriditis antiserum ... 207

B. Phenomena of coagglutination—General References ... 208
24. Absorption of B. paratyphosus B antiserum ... 209
25. Absorption of B. typhosus antiserum ... 209
26. Absorption of a polyvalent antiserum ... 209
27. Intestinal flora. Technic for the examination of a stool specimen ... 210

7th week

28. Precipitins ... 214
29. Erythroprecipitins ... 215
30. Coctoprecipitins ... 215
31. Thermoprecipitin reaction in anthrax ... 216
32. Action of Shiga toxin ... 216

8th week

33. Agglutinins in milk ... 217
34. Leucocytosis ... 217
35. Action of leucocidin from Staphylococcus aureus on leucocytes ... 218
36. The action of macrophages ... 218
37. The action of microphages ... 219
38. Reticulo-endothelial system in relation to antibody formation ... 220
39. Opsonins ... 220
40. Hemopsonin ... 222
41. Bacteriotropins ... 222

9th week

42. Tuberculosis infection in rabbits ... 223
43. Tuberculosis infection in guinea pigs and fowls ... 223
44. Skin test for tuberculosis ... 224
45. Production and fractionation of tuberculin ... 224
46. Distribution of organisms in the buccal cavity ... 225
47. Preparation of diphtheria toxin ... 225
48. Diphtheria anatoxin ... 226
49. Natural immunity of rats to diphtheria ... 226
50. Schick test ... 227
51. Kellogg test ... 228
52. Virulence and toxicity tests for diphtheria bacilli ... 228
53. Standardization of diphtheria antitoxin ... 229

10th week

54. Experimental pyemia ... 230
55. Agglutination of Staphylococci ... 230
56. Staphylolysin ... 231
57. Exotoxin of certain strains of Staphylococcus aureus ... 231

PAGE

58. Antistaphylolysin ... 232
59. Septicemia ... 232
60. Empyema ... 233
61. Streptococcus hemolysin ... 233
62. Effect of surface tension on growth of streptococci ... 234
63. Agglutination of streptococci ... 235
64. Focal infection in the rabbit ... 235

11th week

65. Dick test for scarlet fever susceptibility ... 237
66. Demonstration of agglutinins in the serum of convalescent scarlet fever patients ... 237
67. Production of Streptococcus scarlatinae toxin ... 238
68. Protective value and specificity of antipneumococcus serum ... 238
69. Experimental lobar pneumonia in the rabbit ... 238
70. Iso-agglutinins and iso-hemolysins ... 239
71. Mutual matching of students' sera and corpuscles ... 240
72. Clinical matching of bloods ... 241

12th week

73. Normal hemolysins in dog or goat serum ... 242
74. Properties and titration of complement ... 243
75. Artificial hemolysins ... 244
76. Heterophylic hemolysins ... 244
77. Effect of hemolytic sera in vivo ... 245
78. Bordet-Gengou reaction for B. typhosus ... 247
79. Bordet-Gengou reaction for C. diphtheriae ... 248
80. Anticomplementary property of egg albumin and of anti-egg-white serum ... 248
81. Diagnostic complement fixation in tuberculosis ... 249

13th week

82. Active anaphylaxis in guinea pigs ... 250
83. Passive anaphylaxis produced by rabbit antihorse serum ... 251
84. Anaphylactic shock in the rabbit ... 251
85. Anaphylaxis in vitro ... 252
86. Local anaphylactic reactions. Arthus phenomenon ... 252
87. Desensitization or antianaphylaxis ... 253
88. Hypersensitiveness ... 253
89. Local passive sensitization of the human skin to atopens ... 254
90. Hay fever and drug idiosyncrasies ... 254
91. Preparation of fluid extracts and solutions for use in the diagnosis of atopic conditions ... 255
92. Zootoxin—Cobra venom ... 255
93. Nephrotoxin ... 256

14th week

94. Influence of temperature on natural immunity of the hen against tetanus ... 258
95. Nature of the toxin-antitoxin reaction. Action of antitetanolysin ... 258
96. Tetanus intoxication in animals ... 260
97. Protective power of Cl. botulinum antitoxin ... 260
98. Demonstration of Cl. botulinum in food, and the examination of soil for Cl. botulinum ... 261

15th week PAGE

99. Wassermann reaction ... 262
100. Serum diagnosis of syphilis by precipitation, Kahn test ... 262
101. Organotropism ... 262
102. Chemotherapy and parasitotropism ... 263

16th week

103. Production of Pasteur rabies virus ... 265
104. Experimental herpes ... 265
105. Smallpox. Diagnosis, vaccination, and immunity test ... 266
106. The Weil-Felix reaction in the diagnosis of typhus fever ... 267
107. Bacteriophage phenomena ... 267

PART IV. CLINICAL BACTERIOLOGY

Prepared by J. Stickel

I. General instructions ... 271
II. Study of specimens from eye, ear, mouth, nasal sinuses, respiratory tract, and pleura ... 275
III. Study of specimens from genito-urinary tract ... 277
IV. Study of specimens from gastro-intestinal tract and peritoneum ... 279
V. Study of specimens from the skin and sub-cutis and of tissues, exudates, and fluids removed during surgical operations ... 282
VI. Study of specimens from circulatory system and pericardium ... 284
VII. Study of specimens from central nervous system ... 288
VIII. Preparation of bacterial vaccines ... 289

PART V. STATE HYGIENIC LABORATORY TECHNIC

Prepared by W. H. Kellogg

I. Anthrax ... 299
II. Botulism ... 299
III. Diphtheria ... 301
IV. Dysentery ... 303
V. Gonococcus infection ... 304
VI. Hookworm and other intestinal worms ... 304
VII. Intestinal protozoa ... 305
VIII. Leprosy ... 306
IX. Malaria ... 306
X. Meningitis. (Epidemic) ... 306
XI. Plague ... 308
XII. Pneumonia—Type diagnosis ... 308
XIII. Rabies ... 309
XIV. Rocky Mountain spotted fever ... 310
XV. Syphilis—Kolmer's complement fixation test ... 310
XVI. Tuberculosis ... 319
XVII. Typhoid fever ... 320
XVIII. Typhus fever ... 322
XIX. An outline of the types of specimens required and the procedures usually employed in the laboratory diagnosis of the reportable and quarantinable diseases ... 322

PART VI. OUTLINE OF A COURSE IN MEDICAL ZOOLOGY

Prepared by E. L. WALKER

PAGE

I. Introduction ... 333
II. Methods for diagnosing and preserving animal parasites ... 335
III. Parasitic amoebae ... 338
IV. Leishmaniae ... 341
V. Trypanosomae ... 344
VI. Intestinal flagellates of man ... 347
VII. Intestinal cilliates ... 348
VIII. Coccidia ... 351
IX. Malarial parasites ... 352
X. Trematode parasites of man ... 356
XI. The Cestoda ... 363
XII. The Nematoda ... 368

APPENDIX A

Prepared by P. SCHOENHOLZ

Preparation and mode of application of solutions and stains ... 379

APPENDIX B

Prepared by P. SCHOENHOLZ

Preparation and formulae of culture media ... 403

LIST OF TABLES

1. Range of hydrogen-ion indicators ... 27
2. Hydrogen-ion ranges for the cultivation of organisms ... 29
3. Normal temperature, pulse, and respiration of experimental animals ... 65
4. Haematology of experimental animals ... 66
5. Air bacteriology ... 87
6. Biochemical reactions of the most important non-hemoglobinophilic gram negative rods ... opp. 120
7. Fermentation reactions of certain species of the proposed genus Corynebacterium ... 147
8. Fermentation reactions of the most important genera of tribe Streptococcacae ... 149
9. Determination of pneumococcus types by the agglutination test ... 157
10. Determination of pneumococcus types by precipitin reaction in urine ... 162
11. Fermentation reactions of the more important gram negative Diplococci ... 165
12. The preparation and use of certain agglutinating sera ... 203
13. Specimens from the eye, ear, mouth, nasal sinuses, respiratory tract, and pleura ... opp. 276
14. Specimens from the genito-urinary tract ... opp. 278
15. Specimens from the gastro-intestinal tract and peritoneum ... opp. 280
16. Specimens from the skin and sub-cutis and of tissues, exudates, and fluids ... opp. 282
17. Specimens from central nervous system ... opp. 288
18. Gram negative rods which grow on plain blood agar plates ... 293
19. Gram positive cocci which grow on plain blood agar plates ... 294
20. Gram positive rods which grow on plain blood agar plates ... 296

Index ... 427

Special Laboratory Rules for Safety

The Safety of Every Worker in the Laboratory Depends upon the Observance of these Rules

1. Hang wraps, coats, hats, etc., on the hooks provided for this purpose in the hall; never lay or hang them on the desks.

2. Students should enter the laboratory in clean white coats. Before being sent to the laundry these coats should be sterilized in the autoclave. When highly infectious material has been worked with, coats should be disinfected before the student leaves the room.

3. Wipe the top of the desk before beginning and after finishing work with the disinfectant provided in order to reduce dust and avoid contamination.

4. The wire used in making cultures should be sterilized in the flame before and after laying it down. Flame the wire vertically rather than horizontally. If it is covered with viscous material, dry at the side of the flame before sterilizing to avoid scattering infectious material.

5. Put non-infectious solid wastes, cotton, paper, matches, etc., into the buckets provided.

6. Cultures may be killed in the autoclave. If on solid media or media which will solidify, empty into the buckets; if liquid, pour down the sink.

7. Boil instruments (scalpel, scissors, and forceps) in a 2 per cent solution of sodium carbonate before putting them away.

8. Experiments on animals must be conducted in the room assigned for this purpose.

9. At the close of the day's work cleanse the hands by thorough scrubbing with soap and water and rinsing in cresol or bichloride of mercury solution.

10. Before leaving the laboratory shut off the gas and water and see that none of your personal apparatus is left out.

11. Food must not be eaten in the laboratory. Learn immediately to keep string, paper, pencils, pins, fingers, and other objects out of the mouth. Do not drink from laboratory ware. Do not moisten labels with the tongue. Do not smoke in the laboratory.

12. In case a living culture is spilled accidentally, notify the assistant and cover it immediately with an efficient disinfectant. A small piece of cotton placed in the liquid may be considered to give warning of danger. The hands should then be rinsed in disinfectant and thoroughly scrubbed in soap and water.

13. In case of personal accidents, such as cutting or pricking the fingers, or splashing of culture material in the eye, report to the instructor in charge. Use every precaution against infection.

14. Students attending the laboratory exercises must be vaccinated against typhoid and paratyphoid fever.

Rules of the Culture Media Department

1. Special apparatus to replace or supplement that in the locker, or culture media, glassware, special apparatus as burettes, syringes, blood counters, diagnostic sera, etc., needed in special experiments may be obtained from the storeroom on a signed requisition slip. Apparatus returned to the stockroom should be accompanied by a signed slip in order that the student may receive proper credit.

2. Special media requiring preparation should be ordered at least 8 days in advance.

Supplies and Apparatus

Each student will provide the following:

1 copy of Hiss-Zinsser and Russell, *A Textbook of Bacteriology*, New York, Appleton and Company, 1923.

1 copy of Fitzgerald, T. G., *An Introduction to the Practice of Preventive Medicine*. St. Louis, C. V. Mosby Company, 1922.

1 microscope with a substage condenser and $\frac{1}{12}$ oil immersion lens.

Note.—A limited number of microscopes will be assigned to science students. A medical school ruling specifies that the students furnish their own microscopes.

2 laboratory smocks.
1 box microscope slides.
1 box cover slips, No. 1, 22 mm., circles or squares.
1 set dissecting instruments.
1 pair curved-pointed forceps.
1 pair blunt forceps.
1—5 c.c. Luer syringe.
1—2 c.c. Luer syringe.
2—20 gauge one-inch hypodermic needles.
2—23 gauge one-inch hypodermic needles.
2—25 gauge one-inch hypodermic needles.
1 Kolle-aluminum needle-holder.
1 wax pencil for writing on glass.
1 package each of string tags and gummed labels.
1 dozen blotters.
3 towels for drying glassware.
1 old clean handkerchief for cleaning slides.
1 large laboratory notebook (gray chemistry).

Apparatus

Apparatus, lockers, and microscopes will be assigned on presentation of the comptroller's receipt for the laboratory fee. Check the apparatus in locker with the list and return the card properly signed to the stockroom.

LIST OF APPARATUS

Flasks—

1000 c.c.	1
500 c.c.	2
Test tubes	150
Test tubes (Agglutination)	100
Rubber stopper, cap and tubing for safety cap	
Coplin jars	1
Granite funnels	1
Wire baskets	3
Durham tube vials	20

Pipettes—

10 c.c. (grad.)	2
5 c.c. (vol.)	2
1 c.c. (grad.)	5
1 c.c. (vol.)	5
Pipette can	1
Petri dishes	18
Petri dish can	1
Thermometers	1
Planting wires	2
Jar for disinfectant	1

Graduates—

1000 c.c.	1
100 c.c.	1
Hollow ground slides	2
Rubber nipples	3
Glass tubing for pipettes, feet	10
Wire gauze	1
Test tube blocks	2
Slide-holder	1
Granite spoon	1
Granite bowl	1
Tumblers	2
Double boiler	1
Test tube support (copper)	1
Test tube cleaner	1
Ring stand	1
Rings	2
Bunsen burner	1
Tubing (rubber), feet	2
Block for stain bottles	1
Stain bottles	10
Cedar oil, bottle	1
Vaseline, jar	1
Towels	3
Test tube rack	1

Individual apparatus left outside the lockers after the exercise will be confiscated, excepting in the case of microscopes, for which a fine of 50 cents will be collected or levied against the student's deposit. A fee of 10 cents will be charged for the temporary loan of extra keys.

Responsibility for use of sterilizers must be acknowledged by signing bulletins provided; time of starting and release should be shown.

Incubator space is provided in drawers numbered to correspond with the laboratory lockers. Care should be taken not to impede air circulation by cotton placed in the bottom of the drawers.

CLOSE OF WORK

Clean all glassware thoroughly; credit will not be given for dirty glassware.

List everything on hand, compare with posted list, and proceed as follows:

If there is a surplus, return to the stockroom and receive credit.

If short, obtain apparatus from stockroom sufficient to fill out deficiency.

When the apparatus in the locker corresponds with the posted list have one of the laboratory assistants inspect and check each item with the list.

Lock the locker and return the kay to the assistant.

Have the head of your laboratory section examine the microscope in detail, noting any deficiencies in its condition; place the instrument in its case and return the key to the assistant.

No class credit will be given until the business record is complete.

Reading References and their Use

References to current literature are given in the footnotes; consultation of at least some of them, in addition to the textbook reading, is highly recommended in connection with the class exercises, and will be found essential in order to understand and perform the individual problems. Students frequently desire additional references in order to verify a statement, or an instructor casually mentions the name of an author, or a journal, and the approximate year of publication. Instead of aimlessly searching through a number of volumes in order to verify the reference it is recommended that the student learn to consult one of the bibliographic indexes; for example:

Quarterly Cumulative Index to Current Medical Literature.

Index Medicus (main library).

Abstracts of Bacteriology, Baltimore, Maryland.

Chemical Abstracts, Easton, Pa. (Dept. of Chemistry).

Tropical Disease Bulletin, London (Dept. of Zoology).

Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten Referate, Jena.

Centralblatt f. d. gesammte Hygiene und ihre Grenzgebiete, Berlin.

Bulletin de l'Institut Pasteur, Paris.

Excellent summaries and reviews are frequently found in the monographic presentations published in the:

Physiological Reviews, Baltimore (Phys. Lab.).

Medicine, Baltimore, Medical School Library, S. F.

Medical Science Abstracts and Reviews, London.

Ergebnisse der allgemeine Pathologie.

Ergebnisse der Immunitätsforschung.

Monographs of the Rockefeller Institute.

In case a number of students desire to read at the same time an article which has been published in one of the current periodicals, search for a reprint of the article among the following:

Collected Reprints of the Rockefeller Institute for Medical Research.

Collected Reprints of the Hooper Foundation for Medical Research.

Studies from the Otho S. A. Sprague Memorial Institute, Chicago.

Studies in Pathology and Bacteriology, University of Illinois, College of Medicine, Chicago.

Studies from the Department of Pathology, Bacteriology, Hygiene, Cornell University Medical College and several others.

For collateral reading and for the verification of statements presented in the recommended textbook the following publications available in the departmental library will be found exceedingly useful. They are arranged according to subject.

HISTORY OF BACTERIOLOGY

Conn, H. W., and Conn, H. J.
Bacteriology. Ed. 1, Baltimore, Williams and Wilkins, 1923.

Garrison, F. H.
History of Medicine. Ed. 3, Philadelphia and London, W. B. Saunders and Co., 1921.

Cushing, H.
The Life of Sir William Osler. New York, Oxford University Press, 1925.

GENTRAL BIOLOGY OF BACTERIA

Benecke, Wilhelm.
Bau und Leben der Bakterien. Leipzig and Berlin, B. G. Teubner, 1912.

Besson, A.
Practical Bacteriology, Microbiology and Serum Therapy. Trans. from the French by Hutchins. New York, Longmans, Green and Co., 1913. Paris, J. B. Ballière et Fils, 1911.

DUCLAUX, E.

Traité de microbiologie. 4 vols. Paris, Masson et Cie, 1898.

HEIM, LUDWIG.

Lehrbuch d. Bakterologie. Ed. 4, Stuttgart. Ferdinand Enke, 1911.

JORDAN, E. O.

A Textbook of General Bacteriology. Ed. 8, Philadelphia and London, W. B. Saunders and Co., 1925.

KENDALL, A. I.

Bacteriology, General, Pathological and Intestinal. Philadelphia and New York, Lea and Febiger, 1916.

KOLLE, W., AND HETSCH, H.

Die experimentelle Bakteriologie, etc. 2 vols. Ed. 5, Berlin, Urban and Schwarzenberg, 1922.

KOLLE, W., AND V. WASSERMANN, A.

Handbuch der pathogenen Mikroörganismen. 8 vols. Ed. 2, Jena, Gustav Fischer, 1913.

LAFAR, F.

Handbuch d. technischen Mykologie. 5 vols. Jena, Gustav Fischer, 1905–1908.

LÖHNIS, F., AND FRED, E. B.

Textbook of Agricultural Bacteriology. New York, McGraw-Hill Book Company, 1923.

MARSHALL, CH. E.

Microbiology. Ed. 2, Philadelphia, P. Blakiston's Son and Co., 1917.

MEYER, A.

Die Zelle der Bakterien. Jena, Gustav Fischer, 1912.

PARK, WILLIAMS, AND KRUMWIEDE.

Pathogenic Microörganisms. Ed. 8, Philadelphia and New York, Lea and Febiger, 1924.

GENERAL PARASITOLOGY AND MYCOLOGY

BRUMPT, E.

Précis de parasitologie. Ed. 3, Paris, Masson and Cie, 1922.

BUCHANAN, E. D., AND BUCHANAN, R. E.

Household Bacteriology. New York, The Macmillan Company, 1913.

PROTOZOOLOGY

DÖFLEIN, F.

Lehrbuch der Protozoenkunde. Jena, Gustav Fischer, 1911.

HEGNER, R. W., AND TALIAFERRO, W. H.

Human Protozoology. New York, The Macmillan Company, 1924.

MINCHIN.

Introduction to the Study of Protozoa. London, Arnold, 1917.

PROWAZEK AND NÖLLER.

Handbuch der pathogenen Protozoan. Leipzig, Johann Ambrosius Barth, 1912–1925.

INFECTION AND IMMUNITY

COCA, A. F.

Essentials of Immunology. Baltimore, Williams and Wilkins Co., 1925.

BORDET, J., AND GAY, F. P.

Studies in Immunity. New York, Wiley, 1909.

BORDET, J.

Traité de l'immunité dans les maladies infecticuses. Paris, Masson et Cie, 1920.

KOLMER, J. A.

Infection, Immunity and Specific Therapy. Ed. 3, Philadelphia, W. B. Saunders and Co., 1923.

KRAUS AND LEVADITI

Handbuch der Technik u. Methodik der Immunitätsforschung. Jena, Gustav Fischer, 1908–1911.

WELLS, H. G.

The Chemical Aspects of Immunity. New York Chemical Catalog Company, 1925.

ZINSSER, HANS.

Infection and Resistance. Ed. 3, New York, Macmillan, 1923.

ZINSSER, HOPKINS, AND OTTENBERG.

Laboratory Course in Serum Study. Ed. 2, New York, Macmillan, 1921.

CLINICAL ASPECT OF DISEASE

NELSON

Loose-leaf Living Medicine, I and II *and* Medical Service Volume. New York, Thomas Nelson and Sons.

JOCHMANN, G., AND HEGLER, C.

Lehrbuch der Infektionskrankheiten. 2te Auflage. Berlin, Julius Springer, 1924.

CASTELLANI, A., AND CHALMERS, A. J.

Manual of Tropical Medicine. Ed. 3, London, Baillière, Tindall and Cox, 1919.

PATHOLOGY OF INFECTIOUS DISEASES

ASCHOFF, L.

Pathologische Anatomie. 2 vols. Ed. 2, Jena, Gustav Fischer, 1911.

MACCALLUM, W. G.

A Textbook of Pathology. Ed. 5, Philadelphia, Saunders, 1916.

TENDELOO, N. PH.

Allgemeine Pathologie. Ed. 2, Berlin, Julius Springer, 1925.

WELLS, H. G.

Chemical Pathology. Ed. 3, Philadelphia, Saunders, 1918.

EPIDEMIOLOGY AND PREVENTIVE MEDICINE

FITZGERALD, J. A.

The Practice of Preventive Medicine. St. Louis, C. V. Mosby, 1922.

NICHOLS, H. J., AND KELSER, R. A.

Carriers in Infectious Diseases. Baltimore, Williams and Wilkins, 1922.

ROSENAU, M.

Preventive Medicine and Hygiene. New York, D. Appleton and Co., 1923.

VAUGHAN, V. C.

Epidemiology and Public Health. 2 vols. St. Louis, C. V. Mosby, 1922 and 1923.

CLINICAL LABORATORY TECHNIC

LANGERON, M.

Précis de microscopie. Paris, Masson et Cie, 1913.

MALLORY AND WRIGHT.

Pathological Technique. Ed. 5, Philadelphia, Saunders, 1913.

STITT, E. R.

Practical Bacteriology, Blood Work and Animal Parasitology. Ed. 7, Philadelphia, P. Blakistons Son and Co., 1923.

Laboratory Methods of the United States Army (Medical War Manual No. 6). Ed. 2, Philadelphia and New York, Lea and Febiger, 1919.

Preparation of Papers and Theses

Students are required to 'write up' the data of their experimental studies and submit to the instructor a carefully composed paper. In the course on preventive medicine the preparation of a thesis based on a hypothetical epidemiological investigation or a sanitary survey is also required. Experience has shown that in numerous instances the style and the manner of presentation is ineffective and frequently faulty. It is therefore recommended that the student consult one of the recently published books dealing with the preparation of scientific and technical papers before he begins to put his ideas on paper. The best results may be obtained by developing a preliminary outline before the paper is started. As a rule the outline should cover, according to the subject, the headings:

Laboratory Report

I. Title.
II. Object.
III. Equipment and material required.
IV. Method of experimental procedure.
V. Results (observations, data, tables, etc.).
VI. Conclusions: Interpretation of results.

Thesis

I. Title.
II. Introduction: Nature of problem. Review of literature. Object and scope of work.
III. Methods, or explanation of the means by which data were obtained.
IV. Results: detailed description of the experiments.
V. Discussion of results.
VI. Summary.

Citations to the literature may be given in footnotes or they may be placed in the form of a numbered or alphabetically and chronologically arranged bibliography at the end of the paper or thesis. The citations should always include the following items:

> (1) Author's name and initials, (2) the title of the article, (3) the properly abbreviated name of the periodical in which it appeared, (4) the year of publication, (5) the volume number, and (6) the inclusive paging.

Abbreviations of names of periodical publications must be uniform; preference should be given to the abbreviations adopted by the *Journal of the American Medical Association,* which are given in the manual of M. H. Mellish.

References:

Mellish, Maud H. *The Writing of Medical Papers.* Philadelphia and London, W. B. Saunders Company, 1922.

Trelease, Sam F., and Yule, E. S. *Preparation of Scientific and Technical Papers.* Baltimore, Williams and Wilkins Company, 1925.

II. GENERAL LABORATORY TECHNIC

Bacteriologic Glassware

Bacteriologic glassware should have certain physical and chemical qualities. It should be quite resistant to heat to avoid breakage incidental to sterilization. It should also be thick enough to permit moderately rough handling and so obviate the danger of breakage. It should possess a minimum of soluble substances likely to affect the characteristics of bacteria through solution in the culture medium. It should be clear and remain so even after repeated exposure at high temperature so as not to obscure the appearance of cultural features. Needless to say, it should be free from organic matter, free acids or alkalies, and color. It should also be perfectly clean.

A. CLEANING GLASSWARE

Boil in 0.1 per cent lye or washing soda solution; the addition of white washing soap is helpful.

Rinse thoroughly in tap water, then in distilled water. If glassware has been boiled in soda solution rinse in dilute (1 per cent) HCl, then in tap water, and finally in distilled water a few minutes. For special purposes, a cleaning solution may be useful.

Potassium biochromate	40 grams.
Cold water	750 c.c.

When completely dissolved add *slowly* 230 c.c. of concentrated H_2SO_4.

Rinse in hot tap water, then in distilled water, and dry by draining.

Specially clear glassware may be had by wiping each piece dry with a clean cloth. This should always be done in the case of Petri dishes, of slides, and of cover glasses.

The foregoing applies to new glassware. Glassware containing cultures should be sterilized in the autoclave at 15 lbs. pressure for from 1 to 2 hours, depending on the nature of the infected material. The media should be poured out hot, and the glass may then be treated as new glass. Glassware containing whole milk, butter, or fatty substances should be washed separately to avoid the difficulty of removing these substances. This applies especially to glassware having paraffin or vaseline on it; these are best removed by treating the glassware with xylol before boiling it in soda solution.

Vessels which have been used in the preparation of media must be cleaned immediately after using.

To clean new pipettes:

1. Place the pipettes in a convenient receptacle, filled with soapy distilled water.

2. Boil vigorously for 20 minutes over a Bunsen flame.

3. Rinse the pipettes in running water by means of a short length of rubber tubing attached to the faucet, then rinse in distilled water, and drain.

Infected pipettes may be placed in a jar of disinfectant, e.g., $HgCl_2$ 1-1000 with 0.5 per cent HCL, for a few hours and then treated as directed above or they may be kept in a 5 per cent lysol solution.

Microscopical slides.—The slides in general use measure 3 inches by 1 inch, or 76 by 26 mm., and should be of good white crown glass, with round edges.

New slides should be allowed to remain for some hours in alcohol containing 5 per cent hydrochloric acid, then rinsed with running water, thoroughly drained on a towel, dried, and finally polished with old linen.

If only a few slides are required for immediate use, a good plan is to rub the surface with a jeweler's emery paper (Hubert's 00). A piece of hardwood 76 by 26 mm. with a piece of this emery paper gummed tightly around it is an exceedingly useful article on the microscope bench. If the slide is perfectly clean a drop of water may be spread over its surface in a thin even film; if it is greasy, the water collects into small drops and a film cannot be made.

Cover slips.—The most useful sizes are the 19 mm. squares for ordinary cover-glass film preparations and 38 by 19 mm. rectangles for blood films and serial sections; both varieties must be of "No. 1" thickness, which varies between 0.13 and 0.17 mm., in order that they may be available for use with the high-power immersion lenses. In case slides are greasy they should be boiled first in NaOH. They may be cleaned by placing them in cleaning solution. The solution is contained in an evaporating dish or beaker and boiled. The cover slips should be dropped into the fluid one by one. After boiling they are well washed in distilled water and stored in a stoppered jar in 50 per cent alcohol. Before use they are dried with a soft clean cloth.

Used slides and cover glasses should be treated with turpentine or benzol to remove the oil, then treated as new ones. All slides and cover glasses should be carefully wiped. They need not be sterilized. They should be handled only by the edges and may be kept ready for use in a clean tumbler covered with a Petri dish.

B. PREPARATION FOR STERILIZATION

Flasks and culture tubes should be plugged. There are two types of plugs, rolled and pushed.

Rolled plugs are best, but take longer to make.

Pushed plugs are easier to make and adequate for ordinary purposes. Both types will be demonstrated. In a standard tube, 15 cm. by 1.8 cm., 2 cm. should extend outside, 2.5 cm. inside.

Large plugs for flasks, etc., should be wrapped in cheesecloth to prevent fraying and should always be of the rolled type.

Durham tube vials should be placed open end downward in plugged culture tubes.

Petri dishes are stacked, cover side uppermost, in the metal can provided. The tops and covers of the Petri dishes should fit comfortably.

Pipettes are plugged comfortable tight at the mouth end only. Place them tip downward in the metal can provided. (Protruding threads of cotton should be singed off.) The case is provided with an asbestos cloth pad to prevent breakage.

Pipettes for purely qualitative purposes, so-called *Pasteur pipettes,* are prepared as follows:

Clean glass tubing having a diameter of about 6 to 8 mm. is cut into lengths of about 35 cm. A slight constriction is made at a distance of about 6 cm. from each end. This is not necessary, but it serves to prevent the cotton plug from descending. Moreover ,the tube may be readily sealed at this place. The ends are rounded in the blast lamp. A piece of cotton is pushed into each end of the tube by means of a wire or pointed forceps. They are wrapped in paper, sterilized in the dry heat sterilizer and kept ready for use. From these, whenever desired, pipettes can be made in a few minutes. To make a pipette properly, the plugged sterile tube is heated at the middle in a blast lamp. The broad flame should be used in order to soften as long a piece of tubing as possible (3–5 cm.). When the middle is thoroughly softened the tube is removed from the flame and the two halves drawn slowly apart. A relatively wide, thick-walled capillary is thus obtained, whereas if the tube is drawn out rapidly the resulting capillary will be very narrow and thin walled. The drawn-out portion should be about 40 cm. long. This is then heated in the middle and, as a result, two sealed pipettes are obtained. These tubes are frequently used for the sterile collection of blood. The capacity of such pipettes may be increased by blowing a bulb about 8 cm. from the plugged end.

Capillary pipettes are readily manipulated with *rubber teats*, which should be of the best soft vulcanized rubber, and should fit snugly upon the pipette rendering it air-tight.

Reference:

WRIGHT, SIR A. *Technique of the Teat and Capillary Glass Tube.* London, Constable and Company, 1912.

Preparation of a safety cap for pipettes.—Laboratory infections are occasionally traceable to the handling of pathogenic microorganisms in pipettes which are filled by suction with the mouth and emptied gradually by the admission of air under the ball of the finger. Such accidents can be prevented by the use of a safety cap, recommended by F. L. Gates (*Jour. Am. Med. Assoc.*, 1917, 69, p. 467). Each student is required to construct his own safety cap. It consists of a section of two-hole rubber stopper (No. 1 or 2), 1 cm. thick, over which a flat rubber test tube cap is slipped. It may be cemented in place, although that is not necessary. An L of glass tubing forms an easily sterilized mouthpiece, which may be further safeguarded by a cotton plug. Connection with the pipette is made by a stub of straight glass tube, which projects into the cavity of the cap and carries a short length of heavy rubber stethoscope tubing. To give a stiff resistance to pressure from above, the pipette should be pushed into contact with the short glass tube. The finger, pressing the smooth, thin rubber cap down on the end of this tube, controls the delivery of liquid with perfect sensitiveness.

A diagram illustrating the construction of the cap will be available in the laboratory.

C. STERILIZATION OF GLASSWARE

Sterilization by dry heat—

Hot air is preferable to steam sterilization for dry glassware, involving less breakage and chemical soiling, and leaving the apparatus free from moisture. It must be used for metals subject to rust; it *cannot* be used for culture media, or for the sterilization of cotton filters, bandages, rubber goods, etc.

The operation of the sterilizer will be demonstrated. Each student is expected to include a sketch in his notebook showing his conception of the cross-section of the apparatus. Upon operating the sterilizer, sign the bulletin showing the date and time of operation. For the sake of economy try to have the sterilizer full each time it is run. The temperature should be raised gradually to 170° C., timed for 2 hours at 170° to 180°, and the gas turned off. Do not open the door until the chamber has cooled to below 90° C. for fear of breakage caused by draughts.

Sterilization by moist heat—

(1) *Boiling in a water bath.*—This method has only a limited use in bacteriology, being employed mainly for instruments, pipettes, measuring cylinders, and syringes. Fifteen minutes at 100° C. is sufficient to kill all organisms; spores, however, may resist long exposures, even up to 3–4 hours. It is advisable to sterilize instruments soiled with resistant spores in an oil bath at a temperature of 180°C. for 15 minutes.

(2) *Steam sterilization at 100° C.*—This method is extensively used in bacteriology. An Arnold steam sterilizer is employed. The operation of the apparatus will be demonstrated.

Sterilization is effected in two ways.

(*a*) One exposure for 1½ hours. This may ensure complete sterilization, and may be used for such media as broth or nutrient agar in a laboratory in which no dust nor work with anaerobic bacteria introduces chances of contamination with resistant spores. It cannot be used for nutrient gelatin, as this medium, after prolonged heating, loses its property of solidifying on cooling.

(*b*) Exposure at 100° C. for 20 minutes on each of three successive days. The accepted principle of this 'intermittent' or 'fractional' method of sterilization is that one exposure is sufficient to kill the vegetative forms of bacteria; between the heatings the spores, being in a favorable medium, may pass into the vegetative forms which are destroyed during subsequent heatings. This method may be useful for sterilizing media containing sugars which may be decomposed by higher temperatures or by prolonged heating. It is also employed for the sterilization of gelatin media.

The door should always be opened when time is up and the material being sterilized removed at once to avoid wetting the stoppers with water of condensation. In any case it is always advisable to cover the cotton-wool stoppers of tubes or flasks with two or three layers of heavy paper to avoid drenching.

(3) *Steam at high pressure in the autoclave.*—The principle of the autoclave is that water boils when the vapor pressure is equal to the pressure of the surrounding atmosphere. If, therefore, the pressure be increased inside a closed vessel, the temperature at which the water boils will rise above 100° C., the exact temperature depending on the pressure employed. The autoclave consists of a cylinder of gun metal which is supported by a sheet-iron case. The lid is fastened down by screw clamps, and is rendered air-tight by means of an asbestos washer. The apparatus is furnished with a steam tap and pressure gauge, and also a safety valve, which can be set to 'blow off' at any desired

pressure. The usual pressure employed is 15 lbs. per square inch above atmospheric pressure, making a total of 30 lbs. per square inch absolute pressure. With this pressure water boils at 120° C., and fifteen minutes exposure to this temperature kills all forms of organisms, including the most resistant spores. This method of sterilizing is used for such media as broth or nutrient agar, but is usually not applied in the case of certain sugar media or gelatin, as the former are decomposed, and the latter will not solidify on cooling.

Directions for using the autoclave.—The operation of the autoclave will be demonstrated. Having filled the chamber and signed the bulletin, close the door and open the air and steam escape valves. Turn on the steam, at the same time pulling the door tight with one hand and pounding it gently with the other, as the pressure rises, to make it set tightly. As the autoclave heats, air is forced out of the tap and eventually steam issues. Make sure that all air has been expelled from the cylinder and then turn off the air and steam escape valves. The pressure now rises until it reaches the desired level, when the safety valve opens and the excess steam escapes. Count time from the moment the desired pressure is reached. When sterilization is complete, turn off the steam and allow the autoclave to cool until the pressure gauge indicates that the inside is at atmospheric pressure. Never release the pressure suddenly with liquids; they are superheated and will boil vigorously, blowing out the stoppers. As soon as the door can be pushed in by hand the sterilized material should be removed to avoid wetting plugs by the water of condensation.

Care of Instruments

Instruments, scalpels, scissors, bone forceps, and suture needles are sterilized in a 10 per cent solution of liquor cresolis compositus or kept permanently in a concentrated solution and rinsed in boiling water shortly before use. Metal instruments, as forceps and hypodermic needles, etc., may also be sterilized by boiling in distilled water or a 2 per cent sodium carbonate solution held in a sterilizing bath, e.g., an enameled 'fish kettle.' When ready for use, the tray of instruments is lifted out of the sterilizer and laid on a spread towel which has previously been soaked in 1:1000 solution of bichloride of mercury. *Cutting instruments should not be sterilized by boiling.* Instruments contaminated with very resistant spores are sterilized for 10 minutes in liquid petrolatum held at a temperature of 180° C. and then treated in a strong solution of liquor cresolis compositus.

The Use of the Microscope

It is assumed that the student is familiar with the component parts of the microscope and knows the uses and limitations of the instrument. Care must be taken to preserve the accuracy of the microscope. It should be kept at a uniform temperature, not exposed to the sun or any source of heat, and it must be protected from dust. At intervals the instrument should be cleaned. The oil-immersion objective must be cleaned each day after use by wiping the front lens with fine tissue paper known as 'lens paper.' Oil remaining on the lens front dries and becomes sticky; later it becomes hard and is then difficult to remove. Benzol or xylol must be used. *Do not use alcohol,* as the cement that units the component lenses is soluble in alcohol, and in consequence these lens systems would become disorganized and the objective spoiled. If any oil or balsam is accidentally present on the front lens of the dry objectives it must be removed with lens paper moistened in xylol and the lens quickly dried with a soft cloth.

In order to obtain satisfactory results, the microscope should be used under the best possible conditions, and attention should be paid to the following directions while studying stained specimens:

1. The objectives and eyepiece must be clean.
2. The draw tube must be adjusted to the correct length.
3. The flat side of the mirror must be used for stained preparations.
4. The condenser must be adjusted so that its focal points and the specimen coincide.
5. Employ a good artificial illuminant (gas-filled lamp with a frosted bulb).
6. Focus the specimen with the lower power lens first.
7. Open the iris diaphragm to its widest.
8. See that the field is well illuminated before attempting to focus object.
9. To examine a specimen for any length of time, as when searching for bacteria, adopt a comfortable position.

DARK-GROUND ILLUMINATION

This method renders visible delicate unstained organisms, such as spirochaetes, which cannot be seen in unstained preparations with an ordinary micriscope.

By means of a special condenser the specimen is illuminated only with oblique light. The rays do not enter the tube of the microscope, and, in consequence, do not reach the eye of the observer unless they are 'scattered' as a result of the objects (e.g., spirochaetes) being of

different refractive index from the medium in which they are suspended. If so 'scattered,' the organisms appear brightly illuminated on a dark background.

Three articles are required in order to adapt a microscope for dark-ground illumination:

1. A suitable 'dark-ground condenser.'
2. A suitable illuminant of sufficient intensity.
3. A stop which reduces the numerical aperture of the objective to less than 1.0, if an oil-immersion lens is used.

The condenser.—The concentric reflecting type made by Leitz is available in the laboratory. The object of the special condenser is to focus the light on the object, the path of the rays being such that no direct light passes into the front of the lens. The illustration available in the laboratory shows the path of rays through the concentric reflecting condenser.

The illuminant.—The "Mignon" lamp made by Leitz furnishes for any length of time a light which is constant but not so intense as that of the arc.

The funnel stop.—When the objectives employed for dark-ground illumination have a numerical aperture of more than 1.0 (as in the case of oil-immersion lenses), a special stop to reduce the U. A. to less than 1.0 must be employed. This consists of a small funnel-shaped piece of vulcanite which screws into the objective behind the back lens. The stop is removed by unscrewing and the objective can at once be converted for ordinary use.

The thickness of the slide employed is important. The slides should be 0.9 to 1.1 mm. thick and free from scratches. The object to be examined must be at the focus of the condenser, the focal length of which is about 1.2 mm. If too thick a slide is used, the focus of the condenser will be below the specimen and poor illumination will result; if the slide is too thin, the distance between the condenser and slide is such that a large amount of oil must be employed to make contact.

The Leitz Company furnishes a special slide with cover-slip holder, a so-called 'chamber' for dark-ground illumination.

Reference:

LEITZ, E. *Dark-ground Condenser with Direction for Use.* Pamphlet No. 1013, 1923.

Examination of Cultures and Specimens for the Presence of Micro-oranisms

MACROSCOPIC EXAMINATION

Every *specimen or bacterial culture* should be examined, and the consistency, color, and odor noted.

MICROSCOPIC COLONIES

The bacterial colonies or the appearance of the cultures should be described in accordance with Chester's Terminology (see page 49).

A. Examination of living unstained organisms.
   (*a*) "Hanging drop preparation" to observe motility and morphology.
   (*b*) Moist preparations.
   (*c*) Using dark-ground illumination.

B. Examination of stained films—to determine the morphology and the arrangement of the bacterial aggregations.

C. Negative staining methods with India ink, Nigrosine B, or "Cyanochin."

A. Examination of Living Unstained Organisms

(*a*) "*Hanging drop preparations*" are made either in sterile saline, broth, or in 10 per cent KOH solution by the following method:

(1) The concave area of a hollow ground slide is outlined with vaseline.

(2) A small drop (diameter of 2–3 mm.) of diluent is placed on a clean cover slip which is held by forceps.

(3) A very minute amount of the specimen to be examined is emulsified in the diluent.

(4) The cover slip is inverted and placed on the vaseline ring so that the drop is centered in the hollow and slightly pressed to the slide.

(5) The preparation is then examined with the low- and then with the high-power lens. Moist preparations (not hanging drop) may also be made between an ordinary slide and a cover slip in case hollow ground slides are not available.

To examine the preparation, first focus with the *low-power dry lens.* Close the diaphragm of the substage condenser and use the concave mirror. As soon as the edges of the drop are observed distinctly, focus the preparation with the high-power dry lens, while

looking through the ocular, by means of the fine adjusting screw. Bacteria will be observed if the drop is in the center of the lens. When the drop is out of focus droplets only are seen, which have settled down on the cold walls of the chamber which is saturated with moisture. It is often advisable to focus these droplets and then move gradually into the center of the preparation, to find the bacteria. Frequently, when experimenting in the manner described, the cover slip breaks. This may be avoided by gently moving the slide to feel the pressure. If the accident occurs, the entire slide is put in cresol solution and the lens is cleaned by means of lens paper and then disinfected with a soft linen cloth soaked in cresol solution. The oil-immersion lens may also be used to examine hanging drop preparations. If the droplet is examined with this objective, place a small drop of cedar oil on the cover slip after being focused with the high-power dry lens. Then change to the oil-immersion lens and while looking through the ocular bring it to a focus by means of the fine adjusting screw.

After the specimen has been examined remove the cover slip by means of forceps and place the slide and the cover slip in 5 per cent cresol solution. Sterilize the forceps in a gas flame.

It is essential to distinguish between true motility, where the organism changes its position in the field, and Brownian movement, which is an oscillatory movement possessed by all small bodies (whether living or not) suspended in fluid.

(*b*) *Moist preparations for the study of bacteria in exudates, protozoa parasites, etc., in their natural environment.*—Place on the slide a drop of exudate, or intestinal chyme; cover with a cover slip and outline it with vaseline. Examine as directed for the hanging drop preparation. In order to recognize microörganisms the use of vital dyes, as for example neutral red in a dilution of 1:8000–1:10,000, is highhly recommended. Molds and yeasts are teased in a solution of lacto-phenol and Poirrien blue and examined as moist preparations.

(*c*) *Dark-ground illumination with the oil-immersion objective.*—The microscope furnished with special condenser and funnel stop in the objective is placed in front of the illuminant. The condensing lens of the lamp is adjusted so that a parallel beam of light is obtained. With the plane side of the mirror the light is directed into the dark-ground condenser. Using the low-power (⅔ in. or 16 mm.) objective, focus the surface of the condenser so that the engraved concentric rings on the surface come into view. These rings show the center of the condenser; if the condenser is out of center—as it probably will be—adjust the centering screws so that the rings become concentric with the edge of the field. The accurate centering of the

condenser is of the utmost importance, and the time spent in this manipulation will be amply rewarded by the brilliant illumination obtained. The preparation to be examined must be covered with a No. 1 cover slip, and it is advisable to ring round the cover slip with vaseline to prevent evaporation. Place a large drop of immersion oil on the undersurface of the slide or alternatively on the top lens of the condenser, and a similar drop on the cover glass. Place the slide on the microscope stage, taking care that the top lens of the condenser is well below the slide. Rack up the condenser until oil contact is made between the whole surface of the upper lens of the condenser and the slide; then bring the oil-immersion lens into position so that it touches the oil on the cover slip. Adjust the mirror until the field, though dark, is evenly illuminated. Now carefully focus the specimen. A slight adjustment of the condenser, up or down, may be necessary, and some manipulation of the mirror may also be required. After a little practice an evenly illuminated field with an intensely dark background and brilliantly lighted objects may be obtained with a minimum of trouble.

After use, the condenser and objective should be carefully wiped free from oil. Discarded preparations should be dropped into a covered jar of xylol or benzol kept for the purpose. When a sufficient quantity has been accumulated it will be found that they can be very easily cleaned, as the oil and vaseline have been dissolved off in the benzol.

B. Examination of Stained Films

There are a variety of procedures for preparing stained specimens; the methods depend on the material to be examined. A detailed account of the composition and preparation of stains and their mode of application will be found in Appendix A. The general directions applicable particularly to the microscopic examination of solid materials such as bacterial cultures on agar are as follows:

1. Using a wax pencil, divide a clean dry slide into 3 or 4 sections, the slide being held conveniently by means of a Debrand's forceps.

2. In the center of each division place a small drop of diluent (clean water or saline).

3. *Emulsify a very small amount* of the culture in the diluent and spread over an area of about 1 sq. c. The film should be *very thin, even* and well distributed in the field.

4. *Dry the film.* This process may be hastened by holding the slide, film downward, *high* over a low gas flame.

5. *Fixing* of the film is done either (*a*) by heat, passing the slide, film upward, through the middle of the upper half of the flame three times. (Care must be taken to avoid charring of the film when using this method.) (*b*) By chemicals; a mixture of equal parts of absolute alcohol and either or pure acetone or methyl alcohol, is allowed to act on the preparation for from 5 to 15 minutes. The chemical is poured off and the film air dried.

6. *Staining.* The simple and differential stains ordinarily used in the examination of fixed preparations of bacterial culture are:

(*a*) Dilute carbolfuchsin or safranin.
(*b*) Methylene blue.
(*c*) Toluidine blue.
(*d*) Thionin.
(*e*) Dilute methyl or gentian violet.
(*f*) Gram's stain—a differential stain.
(*g*) Acid-fast stain—a differential stain.
(*h*) Wright's stain—a differential stain.
(*i*) Giemsa stain—a differential stain.
(*j*) Wayson's Methylene blue-fuchsin stain—a differential stain.

Special staining methods are used to demonstrate (1) flagella, (2) capsules, (3) metachromatic granules, and (4) spores.

These and other methods will be practiced in the laboratory as the occasion arises.

In most instances it is best to treat the film with a dilute solution of the stain for longer periods of time; at times, however, concentrated solutions are used. The stains are poured directly or filtered onto the slide. When staining is completed, the dye is washed off with water and the slide placed between two sheets of blotting or filter paper. The drying of the film is completed over the Bunsen flame. Such stained films are examined unmounted with the oil-immersion lens.

C. Negative Staining Methods

In a small drop of India ink or Nigrosin B solution or "Cyanochin" emulsify the specimen or culture to be examined. Spread it out rapidly across a slide in an even and thin film, and allow it to dry and then examine with the high-power or oil-immersion objective. The organisms appear a stransparent, occasionally faintly tinged spaces in the gray or deep blue background. The morphology is excellently preserved.

Cultivation of Bacteria

Bacteria cannot be fully identified in a pathological specimen merely by their morphology and staining reactions except in certain cases, e.g., tubercle bacilli in sputum. It is usually necessary to grow them on artificial culture media in the laboratory, and the resulting growth is termed a *culture*. Furthermore, for purposes of identification it is essential that only one species or type of organism be present in the growth, which is then referred to as a *pure culture*.

In order to establish the etiologic importance of bacteria to a disease or to determine their relationship to the environment, it is necessary to obtain a pure culture and study its biochemical and pathogenic properties. This usually involves two distinct operations:

1. The preparation of suitable, germ-free or sterile artificial culture media.

2. The cultivation of the organism and its isolation from others present in the specimen. Only occasionally ,as by blood cultures, can organisms be grown direct from the body in pure culture.

PREPARATION OF CULTURE MEDIA

Culture media constitute the artificial, chemical, and in part, physical environment provided for the study of bacterial growth in the laboratory. They constitute a source of nitrogen, carbon, and salts in addition to a suitable degree of acidity or alkalinity. The majority of the organisms to be studied are pathogenic, and in order to obtain suitable growths the artificial culture media should approximate to the composition and reaction of the tissue fluid in which these organisms grow. Indeed for certain highly parasitic bacteria it is necessary either to add blood or serum to the culture medium or to grow the organisms actually in human transudate, such as ascitic or hydrocele fluid. The basis for the media ordinarily employed in the study of the common pathogenic bacteria is nutrient broth, the function of the agar or gelatin being merely to solidify it. The first stage in the preparation is the making of one of three things: a watery extract of meat; a watery solution of beef extract; or a digest of liver or blood. To the heated extracts is added, as a rule, digested and uncoagulable protein in the form of commercial peptone (1 to 2 per cent). The salt and sometimes the buffer content is increased by the addition of sodium chloride and dibasic sodium phosphate. When the reaction has been adjusted to its optimum by the method outlined

below, the medium is sterilized in the autoclave. Nutrient agar and gelatin are prepared from the basic peptone broth or digest solution. Both broth and agar media may be enriched or modified by the addition of various substances, as sugars, serum, blood, etc.

Under the designation of "sugars" is included a variety of fermentable substances, chiefly carbohydrates, which are used in the identification and classification of organisms. The substances are fermented upon the formation of acid, and, if the process continues, the acid is split up and gas is formed in addition. The medium consists of peptone, or serum water, or fermented "sugar free" broth, to which the fermentable substances are added in the proportion of 1 per cent. An indicator is added to denote acid change. This may be litmus solution (Kubel-Tiemann), neutral red (0.25 per cent of a 1 per cent solution), Andrade's indicator (1 per cent), china blue-phenol red solution, or bromcresol purple and phenol red solutions (0.1 per cent saturated aqueous solution). In order that gas may be detected, a small inverted vial is placed in each culture tube (Durham's fermentation tube). During the process of sterilization the heat drives out the air from the inverted vials, which should be completely filled with liquid and contain no air bubbles when the tubes are cool. The sugars are always made up separately in 10 or 25 per cent solutions, which are sterilized in the autoclave, or by filtration through a Berkefield No. V filter. The requisite amount of sterile sugar solution is added to the peptone water. The medium is distributed asceptically into tubes provided with colored cotton plugs and steamed for 20 minutes at 100° C.

Reference:

MUDGE. The effect of sterilization upon sugars in culture media. *Jour. Bacteriol.* 1917, 2, p. 403.

The fermentable substances most commonly used are the following:

	Colors of cotton plugs
Monosaccharides:	
(*a*) Pentoses—	
(1) Arabinose (from gum arabic)	pink and black
(2) Xylose (from wood)	blue and yellow
(3) Rhamnose	orange and white
(*b*) Hexoses—	
(1) Glucose	pink
(2) Laevulose	purple
(3) Mannose (from the ivory nut)	blue and white
(4) Galactose (made by the hydrolysis of lactose)	pink and green
Disaccharides:	
(1) Saccharose (cane sugar)	green
(2) Maltose (malt sugar)	brown
(3) Lactose (milk sugar)	white

	Colors of cotton plugs
Trisaccharide:	
Raffinose	black and white
Polysaccharides:	
(1) Starch	black and blue
(2) Inulin (from dahlia tubers)	orange
(3) Dextrin	black
Alcohols:	
(*a*) Trihydric—Glycerol (Glycerin)	yellow and white
(*b*) Tetrahydric—Erythritol (Erythrite)	brown and white
(*c*) Pentahydric—Adonitol (Adonite)	green and white
(*d*) Hexahydric—Mannitol (Mannite)	blue
Dulcitol (Dulcite)	orange and black
Sorbitol (Sorbite)	pink and black
Glucosides (vegetable products which on hydrolysis yield a sugar):	
(1) Salicin	yellow
(2) Coniferin	green and brown
Non-carbohydrate substances:	
(1) Inosite—a benzol compound.	purple and white

The degree of alkalinity or acidity produced in culture media after bacterial growth is most accurately determined by the colorimetric method which is described under "standardization of media."

Aside from the broth, gelatin, and agar preparations the following media are used for general purposes: peptone water, litmus or brom-cresol purple milk, Loeffler's or Greenspoon's blood serum, raw or cooked blood broth or blood agar, serum broth and serum agar, Petroff's glycerin egg medium, potato slopes, beef-heart medium, Sabouraud's medium, Smith-Noguchi medium, etc. For the enrichment and isolation of various pathogenic organisms, so-called special media are used. The procedures and formulae for their preparation will be found in Appendix B.

Standardization of media.—While many bacteria will show vigorous growth within a fairly wide range of reaction as regards acidity or alkalinity, others require the reaction of the medium to be adjusted within narrow limits before multiplication takes place. Moreover, all organisms have a particular reaction at which the growth is optimum or at which it ceases.

In order, therefore, to obtain the best growth, particularly of the highly parasitic organisms, the adjustment of the reaction should be made as accurately as possible. This was formerly done by means of titration, but the essential point in question of reaction of culture media is the hydrogen-ion concentration. For a comprehensive discussion of the principles involved the following publications should be consulted:

CLARK, W. M. *The Determination of Hydrogen-ions.* Ed. 2, Baltimore, Williams and Williams Company, 1922.

CLARK, W. M., AND LUBS, H. A. The colorimetric determination of hydrogen-ion concentration and its application in bacteriology. *Jour. Bacteriol.*, 1917, 2, pp. 1, 109, 191.

A summary will be found in most of the textbooks, in particular Park, Williams and Krumwiede (ed. 8, 1924, pp. 97 ff.).

Two methods are given here: one in which the acidity or alkalinity is expressed in relation to the neutral point of an indicator (phenolphthalein), and the other in terms of the absolute acidity which depends on the H-ion concentration. The latter is the standard method of this laboratory. The titration method is used in a modified form to determine the buffer index of a culture medium.

1. METHOD OF TITRATION TO PHENOLPHTHALEIN

In this method the reaction is expressed as being acid or alkaline in terms of the neutral point of phenolphthalein (P_H 8.2). If acid the prefix + is employed, and the sign — denotes alkalinity. The precise reaction is expressed by the number of c.c. of normal acid or normal alkali that is required per liter of medium in order to adjust it to the neutral point of phenolphthalein. Thus a reaction of +2.0 indicates (by the +) that the medium is acid and that it requires 2.0 c.c. of N/1 NaOH per 100 c.c. tot make it neutral to phenolphthalein.

Material required.—Freshly boiled distilled water, casserole, burette with accurately titrated N/20 NaOH; N/1 NaOH; phenolphthalein solution (0.5 per cent in 50 per cent ethyl alcohol).

Technic of room temperature method.—Put 45 c.c. of distilled water in a casserole. Add 1 c.c. of phenolphthalein solution and 5 c.c. of the medium to be tested (when titrating agar, warm the distilled water to 35° or 40° C., not more). If no pink color is present the medium is acid to phenolphthalein. Read the burette, which contains N/20 NaOH, and record figures. While the mixture in the casserole is being stirred, run in very slowly a little of the solution from the burette until a faint pink (25 per cent red and 75 per cent white as shown by the disks of the standard color top) tinge is observed. This color should not disappear on stirring. Read the burette again and record figures. Determine amount of N/20 NaOH withdrawn from the burette. On this is based the calculation for the adjustment of the reaction of the medium.

Calculation.—Five c.c. of medium required, 2.4 c.c. of N/20 NaOH, therefore 100 c.c. (20 times as much) would require 2.4 c.c. of N/1 NaOH; in other words, the medium is 2.4 per cent acid to phenol-

phthalein or +2.4 if expressed according to Fuller's scale. Assuming that a reaction of + 1.0 per cent is desired, then 2.4 — 1.0 c.c. or 1.4 c.c. of N/1 NaOH to every 100 c.c. of medium or 14 c.c. must be added to a liter.

If, on the other hand, the mixture in the casserole should show a pink color when the phenolphthalein is first added the medium is alkaline to this indicator. To determine the degree of alkalinity by the titrimetric method N/20 HCl or preferably N/20 lactic acid is used in a burette and the same procedure followed as given above.

Inasmuch as the titrimetric method takes into account only the total or titrable acidity of a solution and gives no data as to the actual acidity, the colorimetric method is now preferred.

2. METHOD OF STANDARDIZATION ACCORDING TO HYDROGEN-ION CONCENTRATION

Solutions of fixed and known hydrogen-ion concentrations are prepared and to each solution a definite amount of indicator is added. The resulting tint is the standard to which the medium must be brought by titration with alkali, and so the amount of alkali to be added per liter may easily be calculated.

Apparatus required—

(*a*) A set of tubes of standard bore containing solutions of known P_H to which the indicator has been added. These solutions are made up by various buffer solutions. These standard tubes, which have a range from P_H 3.8–9.6 at intervals of P_H 0.2, are made up with the following indicators:

TABLE 1

RANGE OF HYDROGEN-ION INDICATORS

Indicator	Full acid color	Full alkaline color	Sensitive range PH
Bromcresol green	Yellow	Blue	3.8—5.4
Bromcresol purple	Yellow	Purple	5.2—6.8
Bromthymol blue	Yellow	Blue	6.0—7.6
Phenol red	Yellow	Red	7.2—8.8

These tubes are available in the laboratory.

(*b*) Two tubes with distilled water.

(*c*) A comparator block with six holes.

(*d*) Pyrex tubes, which have the same bore and thickness of wall as those containing the standard solutions.

(*e*) Solutions of bromcresol green, bromcresol purple, bromthymol blue (alkaline 0.04 per cent) and phenol red (alkaline 0.02 per cent) in distilled water.

(*f*) A N/1 NaOH solution roughly standardized as follows: 41 gm. of 98 per cent caustic soda dissolved in distilled water and made up to 1 liter. From this prepare very accurately a $\frac{1}{20}$ dilution, the so-called N/20 NaOH solution. (Put 5 c.c. of 4 per cent NaOH in a 100 c.c. volumetric flask and fill up to 100 c.c. with distilled water.)

(*g*) A burette, preferably a micro burette, measuring to 0.01 c.c. (A 1 c.c. graduated pipette may serve in an emergency.)

Technic of adjustment of reaction by colorimetric method.—To save time make the following preliminary test: Place several large, well-separated drops of the diluted medium on a porcelain plate. To each drop add one drop of a different indicator. The resulting colors will serve as guides in the choice of the correct indicator to use for the test. This procedure is called 'spotting.' Put 5 c.c. of the medium to be tested in each of two test tubes. To one tube add 0.5 c.c. of an indicator whose range covers the H-ion concentration of the medium determined by the 'spotting' test, for exammple, P_H 6.0. Compare this tube with the standard tubes marked P_H 6.0 and 6.2. The comparison should be made in the comparator block in which the color of the medium in the tube without indicator is superimposed on the standard tube. If the tube of medium plus indicator matches the standard tube P_H 6.0, the reaction of the medium is P_H 6.0; if the shade of color falls midway between the standard tubes P_H 6.0 and P_H 6.2, the reaction is read as P_H 6.1. If the color matches neither correctly, read to the nearest.

If now the desired reaction of the medium is P_H 7.4, place in the comparator the standard tube P_H 7.4 containing the same indicator in the same amount, add to the media tube, drop by drop from the burette, N/20 NaOH, mixing the contents of the tube after each addition of alkali until the shade of color matches that of the chosen standard tube (P_H 7.4). Final readings should be made at 20° C. or 37° C.

Calculation.—25 $\times$ the amount of N/20 NaOH withdrawn from the burette gives the amount of N/1 NaOH to be added to 1 liter of the medium when 2 c.c. are tested. Example: If 2 c.c. of medium required 0.6 c.c. of N/10 NaOH, 5 c.c. of medium would require $2.5 \times 0.6 = 1.5$ c.c.; therefore, 100 c.c. would require 1.5 c.c. of N/1 NaOH and 1000 c.c. would require 15.0 of N/1 NaOH.

The standardization of a solid medium such as agar presents greater difficulty than in the case of fluid media. It is best to titrate the melted medium and then compare the color when cold.

The culture media used in the laboratory are standardized by this method. In the cultivation of pathogenic bacteria the following optimum reactions should be maintained.

TABLE 2

HYDROGEN-ION RANGE FOR THE CULTIVATION OF ORGANISMS (DERNBY)

	Optimum	Limits
B. typhosus	6.8—7.2	4.5—8.0
B. coli	6.0—7.0	4.5—9.0
B. dysenteriae	6.0—7.2	4.5—8.2
Pneumococcus	7.8	5.0—8.0
Streptococcus	7.6—7.8	4.5—8.0
Tubercle bacillus	6.0—6.5	4.5—8
Diphtheria bacilli	7.2—7.6	5.7—8.2
Gonococcus	7.3	6.0—8.3
B. anthracis	7.0—7.4	6.0—8.5

3. STABILIZATION OF CULTURE MEDIA

After the reaction has been adjusted certain media must be 'stabilized,' i.e., heated at the temperature at which they are to be sterilized in order to precipitate those components that are insoluble at the sterilizing temperature. The length of time and the temperature at which the process is carried out will vary with the amount and type of medium. Final P_H determination should be made after stabilization and before the final filtration and sterilization.

4. CLEARING AND FILTERING OF MEDIA

Liquid media are usually cleared after having been stabilized in the Arnold sterilizer or autoclave by filtration through ordinary filter paper or through "Chardin" filter paper. Agar and gelatin may be cleared by:

(*a*) Sedimentation in tall cylinders. After the heavy particles have settled to the bottom of the cylinder the hardened medium is removed from the container, the turbid portion cut off and discarded. Although this method is somewhat wasteful it is more economical when filtration through cotton or paper must be avoided (hormone agar).

(*b*) Filtration through asbestos glass wool by suction. This method is applicable for the preparation of Liebig's extract or veal infusion agar or gelatin.

(*c*) If exceptionally clear agar or gelatin is required egg white is used. After cooling the agar or gelatin mixture to from 50° to 55° C. the beaten white of one egg is added to each liter of medium; the mix-

ture is then ready to be stabilized. The simplest method of filtration subsequent to stabilization is by means of properly prepared cotton filter pads. They are prepared in the following manner: Between a double layer of cheesecloth about 12–15 inches square two or three layers of absorbent cotton are placed in such a manner that the fibers of one are at right angles to those of the other. The cotton filters are placed in the funnels and wetted with boiling water just before the medium is poured through. Occasionally funnels supplied with hot water jackets may be needed. For routine work, even when large quantities of media are prepared, this procedure is not necessary. Digest agars are always cleared by the last method as the other two described do not give satisfactory results.

After the medium is cleared and the reaction checked it is distributed into the proper containers either in measured or approximate amounts. Flasks, nursing bottles, and all large containers should always be capped either with paper or heavy muslin before the final sterilization. The container is labeled—the amount, kind, and reaction of the media is recorded and the product is then ready for sterilization.

In most instances the basic media are sterilized in the autoclave at 15 pounds pressure. The length of sterilization will depend on the size of the container, and the type of media. It should be noted that tubes in baskets should not be packed tightly—that agar media or liquid media in large containers should be placed in the autoclave while still hot, otherwise the time necessary to melt the agar or heat the liquid contents must be added to the time allowed for sterilization. Ordinarily broths in test tubes or in small flasks (not containing solid meat, brain, etc.) are sterilized for 20 minutes at 15 pounds pressure, while media in large containers are sterilized for from 30 minutes to one hour. Details are given in Appendix B.

For the sterilization of carbohydrate media special care is required. The sterile carbohydrate solution should be added to the finished base. Prolonged heating of carbohydrate media results not only in the breakdown of the carbohydrate but also in a change in the reaction of the media. Alkaline carbohydrate media in particular are subject to marked changes in reaction when overheated. This is to be avoided as in many instances the alkalinity is just as desirable as the presence of the carbohydrate.

The carbohydrate may be sterilized in the autoclave for 12–15 minutes at 15 pounds pressure. For lactose and maltose, which are easily broken down during sterilization, filtration through a Berkefeld V candle is preferable. The sterile solution is then added to the base and after sterile distribution into test tubes, nursing bottles, or flasks the media are heated in the Arnold steamer at 100° C. for 20 minutes.

For the majority of the exercises, properly prepared and standardized culture media will be furnished by the culture media department. Their composition, and the method of preparation, will be found in Appendix B.

In the course of the regular laboratory exercise a few days will be devoted to the preparation of simple media. The methods of sterilization, standardization of the reaction, and the tubing of media will be practiced.

5. DETERMINATION OF THE BUFFER INDEX OF CULTURE MEDIA

It is frequently desirable to know the buffer index of a culture medium. The method developed by J. B. Brown, Hydrogen-ions, Titration and the Buffer Index of Bacteriological Media (*Jour. Bacteriol.*, 1921, 6, p. 555), should be used.

6. USE OF CULTURE MEDIA

Depending on the method of inoculation to be used, solid media are solidified in tubes as follows:

(*a*) Upright, for 'puncture' or 'stab' culture.

The test tube is half filled with the medium, which is allowed to solidify in the upright position. It is inoculated by plunging a long straight wire, charged with the material, vertically down the center of the tube. This method is used for anaerobic cultures in glucose agar, and for testing the liquefaction of gelatin.

(*b*) Sloped, for 'stroke' culture. This is often called a 'slope' or 'slant' and insures a maximum surface of the medium exposed to the air. Quantities of 5–8 c.c. of medium for an ordinary test tube are sufficient. When a large number of agar tubes have to be sloped, special trays, which allow the tubes to be laid at the correct angle, are useful. Fresh agar slopes, after cooling, contain "water of condensation" at the foot of the tube, and the tubes should be stored and handled in the vertical position to prevent the fluid from flowing over the surface of the medium.

Plates.—Where a large surface is necessary, as in the separation of organisms from mixtures, the medium—e.g., agar or gelatin—is allowed to solidify in form of a thin layer in a Petri dish. For a dish of 3½ inches diameter, 12 c.c. of medium are ample.

Shake cultures.—Agar or gelatin medium in tubes is inoculated in the melted condition at a temperature which keeps the medium fluid, but is not immediately lethal to the organisms inoculated (45° C.). The contents of the tubes are mixed by rotation between the palms

of the hands and left to solidify in the tube so that colonies may develop in the depth of the medium, as when separating anaerobes. In the latter case two procedures may be used to expose the colonies for 'picking.'

The test tube is filed and broken or the tube is rotated in a flame. As soon as the agar column is loose only the bottom of the tube is held at an angle in the flame. The steam generated will push the whole column of agar into the dish held before the opening of the tube. The colonies may be sucked up with capillary pipettes or the agar column is sectioned with a sterile blade and colonies fished with a needle controlling the work under a dissecting lens.

Fluid media are used in test tubes or flasks plugged with cotton wool and filled according to the quantity of medium required for individual cultures.

Inoculation of culture media.—Acording to the nature of the medium and the inoculum, various methods are employed for inoculation, and the following instruments are commonly used:

Platinum loop.—A piece of resistance or nichrome wire, 26 gauge, 2½ inches long, inserted into a special aluminum holder; the other end of the wire is bent in the form of a loop, care being taken that the loop is flat and completely closed. The wire is sterilized by holding it vertically in a Bunsen flame so that the whole length becomes red hot at the same time.

Long straight wire.—A wire 4½ inches long mounted on a holder. It is employed for deep stab inoculation when working with anaerobes.

Scalpel.—This instrument, sterilized by dipping in alcohol and flaming, is used for making inoculations with scrapings from tissues and ulcers, etc.

Sterile capillary pipettes.—Method of making described on page 13. These pipettes are necessary for inoculating Smith-Noguchi and beef-heart media.

Sterile pipettes (10–100 c.c.) are used when large amounts of fluid inoculum have to be added to a medium.

TECHNIC OF INOCULATING TUBES

The following routine methods are recommended:

Inoculation of one 'slant' from another.—The two tubes are firmly held at their lower ends between the thumb and first two fingers of the left hand, with the sloped surface of the *medium toward the worker.* Always hold the tube slanted, never upright. The tube containing the growth should be on the left and the uninoculated tube on the right.

With the right hand loosen the cotton-wool stoppers by rotating them in the mouths of the tubes so that they may be removed easily. Take the holder of the inoculating wire at its end between the thumb and first two fingers of the right hand (as in holding a pen). Sterilize the wire by holding it vertically in the Bunsen flame. Remove the stoppers of the tubes from which the inoculation is to be made with the crooked third and the little finger of the right hand, and flame the mouth of the tubes. Pass the needle into the tube and touch a portion of the medium free from growth to ascertain if the needle is sufficiently cool. If too hot, the wire will melt the agar, causing a furrow, and would of course kill the organisms in removing the growth. When the wire is cool, the growth is scraped from the surface, care being taken not to wound the agar. Withdraw the wire and flame the mouth of the second tube. Insert the wire charged with the growth and lightly smear the surface of the agar. Withdraw the wire and sterilize it, flame the mouths of the tubes and replace the stoppers. The nature of the inoculated material and also the date should be written on the tube by means of a wax pencil.

With stab cultures, the tubes are similarly held and the straight wire charged with bacterial growth is plunged into the center of the medium, care being taken to withdraw the wire in the same line, and not to cause splitting of the medium.

In inoculating a fluid medium, such as broth, from a solid culture, the tube should be inclined almost to the horizontal and the growth on the loop deposited on the wall of the tube just above the surface of the liquid at the lower end of the tube. It is advisable to emulsify the inoculum in a small amount of medium. On returning the tube to the vertical position the inoculum is now below the surface of the broth.

Separation of Bacterial Mixtures and Purification of Cultures

Several methods may be used to purify cultures:

1. By plating dilutions of the inoculum ('pour plate' method).
2. By plating decimal dilutions of the inoculum.
3. By heating and subsequent plating.
4. By shake cultures in tubes.
5. By successive strokes or spreading ('streak plate' method).
6. By single cell picking.
7. By animal inoculation.

The methods 1 to 5 provide for the dilution of the mixture in suitable culture medium, usually with an agar or gelatin base, incuba-

tion at suitable temperatures, and the isolation of single colonies which develop theoretically from one but very often from more than one cell. These methods will be demonstrated and practiced.

1. *'Pour plate' method.*—Melt 3 tubes of agar in boiling water. Let the agar cool to 42° until the tubes can be held comfortably in the hand. This cooling down is necessary, as otherwise the bacteria which are brought into the liquified media are killed by the high temperature.

When the right temperature is reached, heat the platinum loop in a gas flame, take out the cotton plug from one test tube and hold it between the fingers in such a manner that the plug or the tube end projecting outward does not touch anything during the inoculation process. Infection from the air is less likely. Carefully flame the mouth of the tube, then bring a loopful of the mixture into the agar, which is held in a slightly tilted position. The droplet from the needle is placed inside the test tube at a place where it just reaches the fluid of the tube and the material gently rinsed from the loop. Place the platinum needle, after flaming, back in its stand. Agar melts only at a temperature of 98° C. and solidifies at 37° C. Take care, therefore, when cooling down the agar to 42° C. that the moment for inoculating is selected when the temperature is just above the solidifying point. The bacteria can be brought easily into the media at this moment without danger of killing them. The work can be simplified by leaving the tubes in a water bath until the thermometer reaches approximately 42°; the test tubes are then inoculated and the plates poured. If no thermometer is available, cool the melted agar tubes until they can be held comfortably in the hand. Replace the cotton plug, then mix the droplet by carefully rolling the agar. This brings about uniform distribution of the bacteria. If now a plate is poured, the medium solidifies, the bacteria become fixed *in situ*, and each develops singly, as a colony. In the particular case, the bacteria are so numerous and the colonies so close together, that an isolation would be impossible. Dilutions must therefore be prepared from the first tube (original) into a second (I, dilution) and into a third tube (II, dilution). Flame the loop and transfer three loopfuls of agar from the original tube into the second and mix by *carefully rolling*. Again flame the loop and transfer three loopfuls from the second tube to the third tube and mix as described before. After all the tubes have been inoculated, pour the agar into Petri dishes, first carefully flaming the mouth of the tube, and after quickly cooling, raise the edge of the cover on one side of the Petri dish sufficiently to allow inserting the mouth of the tube. After the agar is poured out, replace the cover immediately.

When 'plating,' the dishes should stand horizontally. The bottoms of the dishes should be uniformly covered by agar: this can easily be brought about by gently rotating the dishes. Label and number the Petri dishes to correspond with the dilutions in the tubes, in the following way:

Mixture or culture, July 19, 1925.
Peptic digest.
Original, I; II (1st dilution); III (2d dilution).

In the same way, two series of gelatin plates, of three tubes each, are made.

Possible Errors: An irregular surface of the agar shows (*a*) the agar was not entirely liquified (not heated long enough), or (*b*) when cooling down the tubes became partially solidified. Agar plates made by this process are commonly used at the present time, instead of gelatin plates, in countries where the room temperature rises over the melting point of the gelatin and no cold incubator is available.

One series of the agar plates is kept in the drawer or in the cabinet of the working table. The other series is incubated at 37° C. The agar plates are generally inverted when they are placed in the incubator to avoid the spreading of the colonies due to falling drops of water of condensation.

2. *Separation by plating decimal dilutions of the inoculum.*—A series of tubes of melted agar or gelatin are inoculated with successive decimal dilutions of the infected material and then poured into Petri dishes and allowed to solidify. By dilution, the bacteria are separated from one another, and on incubation the resulting colonies are distributed singly throughout the solid media, so that individual colonies may be removed and a pure culture obtained.

3. *By heating and subsequent plating.*—This method is employed where the organisms to be obtained in pure culture are more resistant to heat than the remainder of the bacteria present. This method applies especially to spore-bearing organisms, such as the anaerobes, whose spores survive the heating. The mixture of bacteria is heated to 80° C. for half an hour and then plated. The spores form individual colonies, which may then be 'picked off.' When dealing with anaerobes the heated specimen is distributed in deep tubes or the plates are incubated under anaerobic conditions.

4. *By shake cultures in tubes.*—(See p. 31.)

5. *Streak plate method.*—Three tubes of agar containing 12–15 c.c. of medium (or small flasks containing 100–500 c.c. agar if many plates are poured) are heated in a water bath until the agar is completely melted. After cooling the contents to about 45° C. the plates are

poured and allowed to harden. If the agar is poured into the Petri dish when the medium is still hot the steam will condense on the lid. In this case it will be necessary to dry out the plates in the thermostat by inverting them and allowing them to remain at a temperature of 37° C. for from ½ to 1 hour. To avoid this necessity be sure that the agar is cooled before pouring.

After the agar has hardened the lid is removed, placed on the working bench with as little exposure of the inside of the lid to the air as is possible, and the lower part of the dish containing the agar is inverted. In this way organisms from the air cannot fall on to the inside of the lid or on the agar. Three plates are arranged in a row. Flame the loop and charge with a very minute amount of the bacterial mixture, pus, or exudate. Touch the first plate with the infected loop. Flame the loop again. Streak or dally the material over the first plate then on to the second and then to the third. The streaking may be carried out with the loop, a bent heavy nichrome wire, or with a bent sterile glass rod. The plates are then covered, *inverted* and *incubated* at the proper temperature for 24–48 hours or, whenever necessary, for a longer period of time.

After growth has taken place the colonies developing are examined macroscopically, the size, color, shape, texture being noted. The growth is then studied unstained in hanging drop preparation, and in dried preparations stained with simple stains. Care must be taken in *picking off single colonies,* particularly when they are very close to one another, that the point of the needle does not touch any of the neighboring colonies. The plate should first be looked at through the medium by holding it up to the light. The lid should be removed and the dish held round the side by the thumb and middle finger of the left hand. The colonies should be marked by grease pencil rings on the bottom of the dish. To pick off the colony, first sit down with both elbows on the bench. Hold the plate vertically with the left hand, then grasp the holder of the wire like a pen, with the fingers quite close to the wire. Steady the right hand by placing the little finger on the left thumb in the way artists support the hand when painting. The selected colony is then easily removed without touching the others. Lay the plate on the bench, withdraw the right hand to the other end of the holder and inoculate the required medium in the manner previously described. If the colony appears to be all of one type a small amount of it is transferred to an agar slant in which state it may be kept for further study or if it is composed of more than one type it is purified.

Purification is best carried out in the following manner:

(1) Emulsify a small amount of the colony either in sterile saline or peptone broth. Shake gently to separate the clumps and then make

dilution "pour plates." *Do not streak to purify.* Sterile glass beads or sterile sand may be added to help separate the bacterial clumps.

(2) Emulsify a small amount of the growth into peptone broth (a glucose peptone broth), grow for 6–8 hours, and make dilution plates.

(3) For primary isolation and for special purposes purification is best accomplished by the isolation of single microörganisms with the aid of a capillary pipette (Barber's method).

The method is exceedingly delicate, it requires considerable practice and will not be used in the regular exercises. For further information consult the following references:

BARBER, M. A. The pipette method in the isolation of single microörganisms and in the inoculation of substances into living cells. *Philippine Jour. Sci.*, 1914, 9, Sec. B., pp. 307–360.

HELLER, H. H. Principles concerning the isolation of anaerobes. *Jour. Bacteriol.*, 1921, 6, pp. 445–470.

KAHN, M. C. Chamber's micromanipulator for isolation of single bacterium. *Jour. Infect. Dis.*, 1922, 31, pp. 344–348.

(4) *Separation by animal inoculation.*—Advantage is taken of the fact that laboratory animals are highly susceptible to certain organisms, for example, the mouse to the pneumococcus. If a mixture of organisms containing pneumococci—e.g., sputum—be inoculated into a mouse, the animal dies of pneumococcal septicemia, and the pneumococci can be obtained in pure culture from the heart blood. Similarly the plague bacillus can be separated from the contaminating bacteria present in decomposed rat cadavers by scarifying the skin of guinea pigs with the mixture. The plague bacilli will penetrate the skin and produce characteristic lesions from which the organism can be isolated in pure culture.

INCUBATION

Students should familiarize themselves with the mechanism of the incubator whereby any desired temperature may be constantly maintained. The laboratory has an incubator working at 37–37.5° C. and will maintain a 'cool incubator' at 22° C. for the cultivation of fungi and for gelatin cultures. In case the latter is not working, incubate the gelatin tubes in the cabinets of the working tables.

STOCK CULTURES

For the maintenance of cultures for future comparison, so-called "stock cultures," stab cultures have advantages over slants. Delicate organisms, as for example streptococci, pneumococci, etc., are best kept

in sterile, heated (56° C. for 30 minutes) rabbit serum stratified with liquid petrolatum or in modified 'hormone' agar. All stock cultures must be kept at low temperatures with the exception of the meningococci and gonococci, which are held at 37° C. Before using a stock culture for experimental work always test its purity.

Reference:

BAILEY, S. F. "Hormone" mediums. *Jour. Infect. Dis.*, 1925, **36**, p. 340.

METHODS OF ANAEROBIC CULTURE

Anaerobes are usually defined as organisms that will only grow in the absence of free oxygen. They need oxygen, however, for their metabolism which they obtain not direct from the air but from the breaking up of proteins and carbohydrates. Recent work has shown that it is not free oxygen by itself which is inimical to the growth of these organisms but that when molecular oxygen is present, a bacterial peroxide is formed, probably hydrogen peroxide, which prevents their multiplication. Anaerobes may be cultivated, therefore, either by destroying the peroxide as fast as it is formed, by means of catalase derived from fresh animal or vegetable tissue or by preventing the admission of oxygen to cultures.

The following methods are usually employed to establish anaerobic conditions:

(1) Cultivation of the organism in deep glucose-agar media either as stab or shake cultures.

(2) Cultivation in deep glucose broth (long tubes = 8 x ½ inch Noguchi tubes) stratified with sterile melted vaseline. Inoculation is made by means of a capillary pipette after melting the vaseline.

(3) Smith-Noguchi method. Ascitic fluid broth with sterile rabbit kidney and stratified with vaseline.

(4) Cultivation in beef-heart medium, also spoken of as "cooked meat medium" or deep peptone brain medium.

These methods are used for the cultivation of the anaerobes of the gas gangrene group.

(5) For the removal of oxygen from the usual media three procedures are useful.

(*a*) Buchner's method is applicable for media in ordinary test tubes. The test tube containing the medium is placed in a Buchner's tube, a stout-walled tube 8½ inches by 1 inch, with the lower end constricted so that the test tube does not reach to the bottom of the tube. The tube is furnished with a well-fitting rubber stopper. Some solid pyrogallic acid is placed in the bottom of the tube, strong caustic

soda is added, the inoculated tube is quickly introduced, and the rubber stopper immediately inserted. The oxygen is rapidly absorbed and fairly satisfactory anaerobic conditions are obtained.

(*b*) Wright's method, modified by I. C. Hall. Tubes of slanted 3 per cent meat infusion agar with 5 to 10 per cent defribinated rabbit's blood, provided with an absorbent cotton plug, are seeded in dilutions with the material containing the anaerobes. The slanted surface of the agar should be moderately dry. After streaking the slants, the cotton plug is replaced in the usual manner, cut off squarely with the scissors and pushed into the tube so that its top does not touch any part of the slanted medium. The space above the cotton plug is then filled with pyrogallic acid crystals (0.5–1.5 gm.) and a rubber stopper selected of such size as to plug the opening snugly. The tube is then held in the left hand with the inoculated surface toward the right; 1–2 c.c. of 10 per cent lye are poured on the crystals and the rubber stopper placed in position. The tube is then inverted at once in order to avoid soiling the culture with the lye-pyrogallol solution.

Reference:

HALL, I. C. Practical methods in the purification of obligate anaerobes. *Jour. Infect. Dis.*, 1929, 27, pp. 576–590.

(*c*) Burri's method.—The material is distributed by dilution in 3 tubes of deep agar cooled down to 40–50° C. The content of the last dilution is poured on the rubber stopper of a Burri's tube (glass tubing with rubber stopper at one end and cotton at the other), and allowed to solidify, then the second dilution, and finally the seed tube are stratified. The agar column is readily pushed into a sterile dish after the rubber stopper has been removed.

(*d*) Cultivation in the M'Intosh and Fildes jar, modified by Richardson and Dozier.—This apparatus gives excellent results.

The principle is that spongy palladium and spongy platinum acting as catalytic agents cause the slow combination of hydrogen and oxygen to form water. The jar is made of stout glass; the aluminum lid is furnished with a tube and tap, so that hydrogen may be introduced into the jar. Suspended from the lid by means of two wires, which are connected to terminals, is a small glass spool around which is the spongy palladium. This spongy palladium is made by immersing asbestos in a solution of palladium chloride and allowing it to dry; on heating in the blowpipe, the palladium is deposited in a black amorphous spongy layer on the asbestos. A fine coil of resistance wire is wound round the palladiumized asbestos and the ends are connected to the two wires supporting the spool, so that a current can be passed

through and the spongy palladium heated. The spool is surrounded by wire gauze, which, on the principle of the Davy lamp, prevents an explosion of the hydrogen and oxygen mixture.

Petri dishes in the upright position or tubes are placed inside the jar, and also as an indicator a tube containing 10 c.c. of 2 per cent glucose broth, to which 0.1 c.c. of a 1 per cent aqueous solution of methylene blue has been added.

The flange of the jar lid is coated to a depth of about ¼ inch with plasticine modeling clay; the lid is pressed firmly in the clay and the jar is then evacuated through the stopcock. When evacuation of the jar is completed the stopcock is closed and the jar connected to the hydrogen supply (gas tank with reducing valve and pressure control). An electric current of suitable strength (110 volt a.c. circuit reduced by connecting in series with the 3 feet nichrome wire two 50-watt lamps) is passed through the heating coil for 20 minutes. One of the lamps is cut out 2 or 3 minutes after starting to avoid unnecessary heating of the jar and contents. The jar is permitted to remain connected with the hydrogen supply during this operation, after which the stopcock is closed and the jar disconnected for incubation. Within a few minutes the indicator containing the methylene blue should be colorless, showing that complete anaerobiosis is established.

Students are requested not to operate this apparatus without previous demonstration by the instructor.

Reference:

Richardson, A. C., and Dozier, C. C. A safe method for securing anaerobiosis with hydrogen. *Jour. Infect. Dis.*, 1922, 31, pp. 617–621.

Identification and Classification of Unknown Organisms

In practical medical bacteriology, microscopical appearance, chemical reactions, animal and immunological tests are usually of more value than minute descriptions of the appearance of the growth on nutrient culture media. But the medical bacteriologist sometimes encounters bacteria which cannot be identified by the usual diagnostic procedures. Proper classification is then facilitated by using the methods recommended for the study of pure cultures by the Society of American Bacteriologists. It is needless to emphasize that *no organism can be properly classified until the following characteristics have been studied:* morphology, staining reactions and motility; the cultural reactions when grown on various media; the food, oxygen, and temperature requirements, and the action of the organism or its products on experimental animals. In order to record the diagnostically important characteristics a descriptive chart has been prepared by

the Committee on the Descriptive Chart of the Society of American Bacteriologists. These charts can be obtained from the instructor in charge of the section. It is advisable that the student familiarize himself with the glossary of the terms employed on the chart (see below) and the standard methods recommended for the elicitation of the characteristics before beginning the work.

The following is a short summary of the more important steps which must be followed in order to establish all the characters of the organism.

(1) *Preparation of standard media.*—Beef extract agar, gelatin, sugar broth, nitrate broth, indicator media, synthetic media (if necessary), potato, etc. (see Appendix B).

(2) *Classification according to optimum conditions for growth.*—"Before beginning the study of any pure culture, it is important to know something about the growth requirements of the organism. If the organism in question does not grow in ordinary media either because it is an obligate parasite or because it requires the complete absence of oxygen or of organic matter, it obviously cannot be studied by the methods called for on the descriptive chart. For such organisms as these the investigator must use his own methods of study, recording the characteristics in the blank space at the bottom of the back of the chart. Those organisms that do grow on ordinary media have to be studied by different methods according to whether they grow better in liquid or in solid media and according to whether they grow better at body temperature or room temperature. It is important therefore that before studying an unknown culture which is able to grow in laboratory media, these two points in regard to growth requirements be determined. It proves convenient to classify the organisms in this respect into four series. These series can be separated from each other on the lines just mentioned, namely, preference for solid or liquid media, and the optimum temperature for growth. The two media which it seems best to use for this purpose are glucose broth and standard agar, glucose broth being preferred to plain broth to favor those organisms that require a carbohydrate. The temperatures most convenient for use are 37° C. and 25° C. The results are not affected very much if room temperature or 21° be used instead of 25°, but the latter temperature seems to be nearer the optimum for those organisms that do not grow at body temperature. In determining in which series an organism belongs the procedure is as follows:

Series I. Organisms which produce good growth (surface growth, distinct turbidity, or heavy precipitate) in twenty-four hours at 37° in glucose broth.

Series II. Organisms which do not produce good growth in twenty-four hours as the above, but do so in forty-eight hours at 25° in glucose broth.

Series III. Organisms which do not grow well in glucose broth but do produce good growth on the surface of agar in twenty-four hours at 37°.

Series IV. Organisms excluded from the above groups but which produce good growth on the surface of agar in forty-eight hours at 25°.

Record the series number on the chart at the proper place; then if it is felt that the culture in question should be invigorated for study, inoculate into another tube of glucose broth from organisms of series I and II or of standard agar from organisms of series III and IV. Inoculate this tube at the temperature and for the time called for by the series in which it belongs, then transfer from this tube to third tube and incubate as before. The method of invigoration used should be recorded on the chart in the blanks left for this matter."

(3) *Microscopic characteristics.*—Dried preparations preferably made from agar cultures, from a few hours to two days old according to the rapidity of growth, are employed. The medium and temperature used and the age of the culture should be recorded.

Motility.—Hanging drop preparations. If motile or even if motility is not observed microscopic preparations should be made to demonstrate the presence or absence of flagella. Negative results from both hanging drop preparations and flagella stain do not absolutely prove that the organism is immotile.

Presence of endospores.—Routine examination in stained (methylene blue) and unstained preparations; suspend growth in sterile water and heat for 10 minutes at 85° C.; if transplants grow, endospores may be regarded as present. A culture of a large rod should not be recorded as a non-spore former unless all these tests are negative.

Capsules.—An organism should not be recorded as having capsules unless they have been actually stained by one of the methods outlined in the Appendix.

Irregular forms.—They should be noted and sketched; they should not be indiscriminately called "Involution forms." Recent studies of Löhnis and others indicate that some of these forms represent stages in the life-history of certain bacteria.

Special stain.—The Gram stain (ammonium oxalate-gentian-violet) recommended by the Committee should be used judiciously and with the necessary controls.

Drawings of all the microscopic characteristics should be made on the blank spaces on the chart.

(4) *Cultural characteristics.*—Cultures for the study of cultural characteristics should be incubated at 37° C. in case of organisms of series I and III, and at 25° in case of organisms of series II and IV, except that gelatin cultures should be incubated at 20°. Room temperature may be used in place of 25° at certain seasons of the year but if a minimum thermometer shows that the temperature falls below 22° during the course of the work, note should be made of the fact. On the day when good growth first appears the proper descriptive terms on the card should be underlined; after subsequent study, any changes should be noted.

Streak cultures on sugar, potato, blood serum or other solid media. Is the growth abundant or scanty, flat or raised, opaque or transparent, smooth or wrinkled? Is it restricted or spreading? What is the character of its margin?

What is the color?

Has the color, consistence, or odor of the medium changed?

Is the water of condensation clear or cloudy? Is there pellicle or sediment?

Note the characteristics of isolated colonies, when present.

Stab cultures.—Does the growth appear along the line of puncture alone, at the surface alone, or in both places?

Is it confined to the point of puncture; does it spread over the whole surface; is its margin regular or indented; is it flat or raised; dry or moist; what is its color?

What is the character of the growth along the line of puncture?

Is liquefaction present?

Method of determining liquefaction of gelatin.—Inoculate a gelatin stab (plain gelatin) and incubate 6 weeks at 20° C., provided the organism under investigation will grow at that temperature. Care must be taken to observe whether the organisms produce rapid and progressive liquefaction or merely slow liquefaction not extending far from the point of inoculation. In the latter case the liquefaction may be due merely to endo-enzymes that are released from the cell after death and may not be what is generally called "true-liquefaction' (that is the process resulting from ecto-enzymes).

In case the organism under investigation will not grow at 20° C. it may be incubated in gelatin at 37° or whatever temperature may be the optimum, and then after incubation the tubes may be placed in a cold water bath or in a refrigerator to determine whether or not the gelatin is still capable of solidifying.

Growth in liquid media.—Does the broth remain clear, or does it become uniformly clouded, or clouded only at the bottom? Is the sediment granular, flaky, gelatinous, or stringy?

Is the pellicle tenacious or easily broken, spreading up the side of the tube or not, thick or thin, stalactite formation?

Note any change in the color, odor and reaction.

Plate cultures.—With hand lens, note size, shape and color of the colonies, both surface and deep.

Place plate culture in Petri dish on stage of microscope, remove cover and examine with number three objective.

Note form, surface, edge, and internal structure of colonies. Note differences between the surface and deep colonies of the same bacteria.

Bromcresol purple or litmus milk.—Is the milk curdled (acid curd, rennet curd), i.e., coagulated, partially or completely? Is there indication of gas formation, as shown by holes in the curd? Recognize different degrees of acidity, reduction of indicator, proteolytic action.

(5) *Physiology. Relation to free oxygen.*—Two methods should be used.

(*a*) Determine by noting the presence or absence of growth in open and closed arm, respectively, of fermentation tubes containing *glucose* broth.

(*b*) A tube of about 10 c.c. glucose agar in fluid condition (45° C.) is inoculated and cooled promptly in a water bath; colonies of strict anaerobes fail to grow in the upper layers of the medium.

Growth only in free oxygen, obligate aerobe; growth only where oxygen is absent, obligate anaerobe; growth under both conditions, facultative anaerobe; growth with a definite but lessened amount of oxygen (development only in one level of solid media), microaerophilic.

Fermentation of sugars.—Several types of media may be chosen. The sugars selected will depend on the organism to be identified. When studying a series of unknown organisms it is advisable to inoculate all into the prescribed sugar medium (beef extract broth or synthetic medium) with Andrade's or bromcresol purple indicator; later those that show acid may be reinoculated onto the same medium with bromcresol green; and subsequently those positive to this indicator upon the same medium with bromchlor-phenol-blue. These indicators should be added in the concentrated of 1 c.c. of a saturated equeous solution to 1 liter of carefully adjusted media. In the case of liquid media without indicator, the examination can be made very accurately by withdrawing a 2 c.c. sample and comparing it in small tubes with

buffer standards, with the proper indicator, by the method described on page 42. Cultures should be incubated at 37° C. with organisms of series I and III and at 25° C. with organisms of series II and IV. Examine after 24 hours and then as often as seems necessary, according to the rapidity of growth, so as not to overlook any changes in reaction. Gas production in liquid media is ordinarily measured in percentage of gas in the closed arm of the fermentation tube. To determine roughly the proportion of CO_2 and H_2, mark on the tube the quantity of gas produced, then fill the bulb with 10 per cent NaOH and insert a rubber stopper; the tube is inverted several times to mix the fluids and the gas again collected in the closed arm. The gas absorbed is CO_2; the remainder is usually H_2.

Reduction of nitrates.—The following procedure is recommended: Inoculate first into nitrate broth and on to slants of nitrate agar (containing 0.1 per cent KNO_3 plus beef extract and peptone as usual). Test the cultures on various days as indicated on the chart. On these days examine first for gas as shown by foam in the broth or by cracks in the agar. Then test for nitrate with the special reagents (Appendix A).

Put a few drops of each of these reagents in each broth culture to be tested, and on the surface of each agar slant. A distinct pink or red in the broth or agar indicates the presence of nitrite. It is well to test a sterile check which has been kept under the same condition to guard against errors due to absorption of nitrite from the air. Presence of nitrite or of gas shows the nitrate to have been reduced. A negative result does not prove that the organism is unable to reduce nitrates; in such a case further study is necessary as follows:

In case the fault seems to lie in poor growth, search should be made for a nitrate medium in which the organism in question does make good growth by means of the following modifications: increasing or decreasing the amount of peptone; altering the reaction; adding some readily available carbohydrate. Presence of nitrite in any nitrate medium whatever should be recorded as nitrate-reduction. Presence of gas should be similarly interpreted, provided there is no other substance (e.g., sugar) present in the medium from which the organism under investigation is able to produce gas.

In the case of those organisms which are nitrite-negative and produce no gas in nitrate-peptone agar or broth, but which show good growth in one or both media, proceed as follows before concluding that the organisms do not reduce nitrate:

Inoculate into:

A. Nitrate-peptone agar or broth.

B. Peptone agar or broth without nitrate.

C. Synthetic nitrate medium (formula recommended: KNO_3 1 gm., K_2HPO_4 4.0 gm., $CaCl_2$ 0.5 gm., glucose 10 gm., distilled water 1000 c.c., with or without agar according to the organisms under investigation).

D. Peptone agar with 2 p.p.m. potassium nitrite.

Prepare the following reagents for the Thomas test for ammonia:

1. A 5 per cent solution of phenol.

2. A solution of sodium hypochlorite containing one per cent available chlorine. To obtain this amount of available chlorine, the solution should be so adjusted that 1 c.c. of it should neutralize 2.86 c.c. of a N/10 solution of sodium thiosulphate (i.e., 24.8 grams to the liter), titrating with starch as an indicator in the presence of acetic acid and potassium iodide.

In making the Thomas test 1 c.c. of each of the reagents noted should be added to the broth or agar slant* on which the culture has been growing and allowed to stand half an hour. A blue color indicates the presence of ammonia.

After incubation, test A and B for ammonia by the Thomas method; test C for nitrite as usual, and for ammonia by the Thomas method; test D for nitrite. The results give the following indications:

On A. Presence of ammonia indicates nitrate reduction if no ammonia is present in B. Absence of ammonia on both media indicates non-reduction, provided this conclusion is confirmed by the results on C and D. Otherwise inconclusive.

On C. Presence of either nitrate or ammonia indicates nitrate reduction. Absence of both, if the growth is good, strongly indicates non-reduction.

On D. Presence of nitrite indicates non-reduction in case this is confirmed by the two foregoing tests, for if the organism cannot destroy the extremely small amount of nitrite present in D it is not likely to have destroyed the nitrite in nitrate agar as fast as it produced it.

The organisms for which inconclusive results must be recorded are those which produce ammonia in B, which fail to grow on C, and which destroy the nitrite in D. The number of organisms in this group is probably very small.

The evolution of gas (free N) in a nitrate medium containing no sugar or other similar source of gas is a definite indication of reduction. This complete reduction is known as denitrification in the strict sense of the term. It can best be detected by the use of fermentation

* This test can be made for this purpose to great advantage with agar slant culture as well as with liquid media, as recently shown by Hucker and Wall (1922).

tubes for those organisms that grow best in liquid media (series I and II) and in shake cultures of agar for those that do not grow well in liquid media (series III and IV). It is possible in most cases to get indication of gas production by ordinary test tubes of nitrate broth in the case of organisms of series I and II and by agar slants in the case of organisms as series III and IV; but in neither case is the test as delicate.

Chromogenesis.—Color production should be recorded if observed in broth, on beef extract agar, gelatin, or potato, or if noticed to a striking extent on any other medium. In the margin the space devoted to chromogenesis refers to the color produced on beef extract agar.

Hydrolysis of starch.—Use beef extract agar containing 0.2 per cent of soluble starch. Pour into a Petri dish, and after hardening make a streak inoculation on its surface. Incubate at 37° for organisms of series I and III at 25° for organisms of series II and IV. Observations are to be made on the second day for rapidly growing organisms but not until the seventh day for more slowly growing ones. To make the test, flood the surface of the Petri dishes with a saturated solution of iodine in 50 per cent alcohol. The breadth of the clear zone outside of the area of growth indicates the extent of starch destruction.

Indol production.—Use Dunham's (Witte's) peptone solution or the following medium:

To 1 liter of ordinary peptone bouillon add 0.2 gram of trypsin, then chloroform and toluol to prevent bacterial growth and incubate 24–48 hours at 37° C., subsequently filtering and diluting with 3 parts of physiological salt solution. Inoculate and test for indol production on the 2nd and 5th day by the Salkowski method, the Vanillin test, and the Ehrlich test (Appendix A).

Production of hydrogen sulfide.—Use the lead acetate agar method.

Acetyl-methyl-carbinol reaction.—Use method given in Appendix A.

Brief characterization.—On the right-hand margin of the chart there is a space for recording briefly the important characteristics of an organism. In the old chart and the instruction folder the characteristics were recorded by + and — signs; but in the new standard chart this method of description has been much changed, and calls for the use of figures instead of + and — signs. In order to avoid the use of a separate sheet as a key to the significance of these figures, their meaning is given on the face of the chart beside the space in which the numbers are to be set down. The margin of the chart when completely filled out may be recorded as a summary of the important characteristics of the organisms in question.

The characteristics listed under this brief characterization are divided into two main headings: primary characteristics and sec-

ondary characteristics. These are divided into three separate headings: microscopic features, miscellaneous biochemical reactions, and carbohydrate reactions. Under the microscopic features are spaces for recording four figures denoting respectively, form, presence and position of spores, presence and arrangement of flagella and, lastly, behavior with the Gram stain. Under the second heading are spaces for five figures, the first denoting whether the organism is pathogenic, saprophytic or autotrophic, and the other four denoting respectively, relation to oxygen, gelatin liquefaction, nitrate production, and chromogenesis. Under the third heading are four spaces for recording figures, the first denoting diastatic action and the other three respectively, fermentation of dextrose, lactose, and sucrose. These altogether give thirteen figures which, if it is desired to file the cards according to the characteristics of the organisms, may be used as an index number, placing them in a blank left for this purpose near the top of the card. The index number, if thus used, should be separated by points or dashes into the three groups of figures just listed. Thus *B. coli* has the index number 5312–42220–1111.

After a complete study of all the characters of the organisms has been made, and the chart completely filled out, read carefully page V of Bergey's *Manual of Determinative Bacteriology* (Baltimore, Williams and Wilkins Company, 1923). Then turn to page 29 and ascertain first in which order the organism belongs. Later establish the family or subfamily, the tribe, and finally the genus to which the organism belongs.

During the course *each student must study at least five unknown organisms, fill out the descriptive chart and identify the bacteria with the aid of the Determinative Manual.* The instructors will render assistance only provided the reactions are carefully recorded and the chart is completely filled out.

GLOSSARY OF TERMS USED ON THE DESCRIPTIVE CHART

Acid curd, coagulation of milk due to acid production.

Adherent, applied to sporangium wall, indicates that remnants of sporangium remain attached to endospore for some time.

Aerobic, growing in the presence of free oxygen, strictly aerobic growing only in the presence of free oxygen.

Amorphous, without visible differentiation in structure.

Anaerobic, growing in the absence of free oxygen; strictly anaerobic, growing only in the absence of free oxygen; facultative anaerobic, growing both in presence and in absence of oxygen.

Arborescent, branched, tree-like growth.

Autotrophic, able to grow in absence of organic matter.

Beaded (in stab or stroke culture) separate or semiconfluent colonies along the line of inoculation.

Bipolar, at both poles or ends of the bacterial cell.

Brittle, growth dry, friable under the platinum needle.

Butyrous, growth of butter-like consistency.

Capsule, a gelatinous envelope surrounding the cell membrane of some kinds of bacteria.

Chains, four or more bacterial cells attached end to end.

Chromogenesis, the production of color.

Clavate, club-shaped.

Compact, refers to sediment in the form of single fairly tenacious mass.

Concentrically ringed, marked with rings, one inside the other.

Contoured, an irregular, smoothly undulating surface, like that of a relief map.

Crateriform, a saucer-shaped liquefaction of the medium.

Cuneate, wedge-shaped.

Curled, composed of parallel chains in wavy strands, as in anthrax colonies.

Diastatic action, conversion of starch into simpler carbohydrates, such as dextrins or sugars, by means of diastase.

Echinulate, a growth along line of inoculation with toothed or pointed margins.

Effuse, growth thin, veily, unusually spreading.

Endospores, thick-walled spores formed within the bacterial cell; i.e., typical bacterial spores like those of *B. anthracis* or *B. subtilis.*

Entire, with an even margin.

Erose, border irregularly toothed.

Excentric, slightly to one side of the center, between the positions denoted central and subterminal.

Filaments, applied to morphology of bacteria, refers to thread-like forms, generally unsegmented; if segmented to be distinguished from chains by the absence of constrictions between the segments.

Filamentous, growth composed of long, irregularly placed or interwoven threads.

Filiform, in stroke or stab cultures, a uniform growth along line of inoculation.

Flaky, refers to sediment in the form of numerous separate flakes.

Flocculent, containing small adherent masses of bacteria of various shapes floating in the culture fluid.

Fluorescent, having one color by transmitted light and another by reflected light.

Granular, composed of small granules.

Hydrolysis of starch, destruction of starch by the formation of a chemical union with water; includes diastatic action, but is more general term.

Infundibuliform, in form of a funnel or inverted cone.

Iridescent, exhibiting changing rainbow colors in reflected light.

Lobate, having the margin deeply undulate, producing lobes (*see* undulate).

Maximum temperature, temperature above which growth does not take place.

Membranous, growth thin, coherent, like a membrane.

Minimum temperature, temperature below which growth does not take place.

Mycelioid, colonies having the radiately filamentous appearance of mold colonies.

Napiform, liquefaction in form of a turnip.

Opaque, not allowing light to pass through.

Opalescent, resembling the color of an opal.

Optimum temperature, temperature at which growth is most rapid.

Papillate, growth beset with small nipple-like processes.

Parasitic, deriving its nourishment from some living animal or plant upon which it lives and which acts as host; not necessarily pathogenic.

Pathogenic, not only parasitic, but also causing disease to the host.

Pellicle, bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.

Peptonization, rendering curdled milk soluble by the action of peptonizing enzymes.

Peritrichiate, applied to the arrangement of flagella, indicates that they are distributed over the entire surface of an organism.

Peritrichic, having flagella in peritrichiate arrangement.

Persistent, lasting many weeks or months.

Photogenic, glowing in the dark, phosphorescent.

Polar, at the end or pole of the bacterial cell.

Pulvinate, decidedly convex, in the form of a cushion.

Punctiform, very small, but visible to naked eye; under 1 mm. in diameter.

Radiate, showing ray structure.

Raised, growth thick, with abrupt or terraced edges.

Rapid, developing in twenty-four to forty-eight hours.

Reduction, removing oxygen from a chemical compound. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen; also to the decolorization of litmus.

Rennet curd, coagulation of milk due to rennet or rennet-like enzymes, distinguished from acid curd by the absence of acid.

Rhizoid, growth of an irregular branched or root-like character, as *B. mycoides.*

Ring, growth at the upper margin of a liquid culture, adhering to the glass.

Rugose, wrinkled.

Saccate, liquefaction in form of an elongated sac, tubular, cylindrical.

Saprophytic, unable to grow in the absence of organic matter, i.e., not autotropic; but not parasitic, as a living host is unnecessary.

Slow, requiring five or six days for development.

Spindled, larger at the middle than at the ends. Applied to sporangia, refers to the forms frequently called clostridia.

Sporangia, cells containing endospores.

Spreading, growth extending much beyond the line of inoculation, i.e., several millimeters or more.

Stratiform, liquefying to the walls of the tube at the top and then proceeding downward horizontally.

Subterminal, situated toward the end of the cell but not at the extreme end, that is between the positions denoted excentric and terminal.

Transient, lasting a few days.

Translucent, allowing light to pass through without allowing complete visibility of objects seen through the substance in question.

Truncate, ends abrupt, square.

Turbid, cloudy with flocculent particles; i.e., cloudy plus flocculence.

Umbonate, having a button-like, raised center.

Undulate, border wavy, with shallow sinuses.

Villous, having short, thick, hair-like processes on the surface, intermediate in meaning between papillate and filamentous.

Viscid, growth follows the needle when touched and withdrawn; sediment on shaking rises as a coherent swirl.

III. ANIMAL EXPERIMENTATION AND AUTOPSY NOTES ON EXPERIMENTAL ANIMALS

Spontaneous Infections in Laboratory Animals

Animals frequently will be used by students for experimental purposes. It is therefore deemed advisable to give some information concerning breeding and feeding, as well as the spontaneous infections and parasitic invasions which may be encountered in the animal house. The usual animals employed for bacteriological experiments are the guinea pig, rabbit, mouse, rat, pigeon, and fowl.

Guinea pig. These animals vary in size and weight from 200 grams to 800 grams. A good average weight for general purposes is 250 to 400 grams. Large male animals are used for cardiac bleeding (complement); white-colored animals are preferred for skin tests. Short-haired guinea pigs are usually more suitable than the angora type. Males and females at least 7 to 8 months old are used for breeding; the gestation period varies between 60 and 65 days. The newborn guinea pigs (1st litter 1–2; subsequent litters on the average 2–3 animals) are fully covered with hair and have long incisors; they may be separated from their mothers on the 4th to 6th week.

Feeding.—During the summer months, clover, grass, hay, cabbage leaves, and barley or oats; in winter, hay, cabbage leaves, carrots or carrot tops or any other available green food from the market, and grains are offered. Water is needed only when the feed is very dry. Wet or warm stacked green feed frequently produces intestinal disturbances. Guinea pigs kept on concentrated dry feed develop scurvy.

Spontaneous infections.—Pneumonia caused by pneumococcus, *B. bronchisepticus;* acute and chronic pseudotuberculosis caused by *B. aertryke* (Savage and White, 1925) and *B. pseudotuberculosis rodentium* (Pfeiffer); septicemia caused by Pasteurella; epizootic lymphadenitis (lumps) caused by hemolytic streptococci; tuberculosis; epizootic abortion caused by streptococci; filterable virus disease of Petrie and O'Brien; paralysis caused by a filterable virus (Römer).

Reference:

Holman, W. L. Spontaneous infection in the guinea-pig. *Jour. Med. Res.*, 1916, 35, pp. 151–183.

Parasitic Invasions and Diseases

Ectoparasites:

Lice: Trimenopon jenningsi, Menopon extraneum, Gliricola porcelli, Gyropus ovalis.

Endoparasites:

Intestinal tract:

Protozoa: **Amoeba coli*** (Walker), A spinosa, **A. cobayae**, Balantidium caviae(?), Trichomonas caviae, **Eimeria stiedae**, E. jalina.

Nematoda: **Trichinella spiralis** (Hall, p. 10), Gongylonema neoplasticum (Hall, p. 193).

Lungs: Larval forms of ascaris and hookworm by experimental infection.

RABBIT. These animals are often unsatisfactory for experimental purposes owing to their liability to parasitic and intercurrent infections. It is always better to use rabbits obtained from a reliable breeder where the conditions of the stock are known, than to purchase rabbits casually and indiscriminately at retail. The rabbit is of value not so much in diagnostic work as in its usefulness for experimental purposes. It is most extensively employed for the production of immune sera, such as agglutinating and hemolytic sera, which are constantly used in routine laboratory diagnosis.

Fancy breeds are highly susceptible to spontaneous infections and preference should always be given to the short-haired types, which mature in from 7 to 9 months. For skin tests albinos should be chosen. The gestation period varies between 31 and 35 days. The newborn rabbits (usually 3 to 10) are hairless; they are capable of seeing about the 10th day and begin to eat independently from the 14th to the 25th day.

Feeding.—In addition to the feed indicated for guinea pigs, rabbits should receive a small amount of fresh water when grain and hay are given, and from time to time salt should be offered to them.

Spontaneous infections.—Rhinitis (snuffles), pyemic subcutaneous abscesses; otitis interna; sinusitis; pericarditis, pleurisy, and bronchopneumonia caused by *B. leptisepticum* (Webster, *Jour. Exper. Med.*, 1924, 39, pp. 836–877); septicemia, abortion caused by *B. aertryke* (Meyer and Litch), *Jour. Infect. Dis.*, 1921, 28, p. 27; *B. necrophorus* infection (Schmorl), puerperal polybacterial infections; veneral spirochaetosis caused by *Treponema pallidum* var. *cuniculi* (Noguchi, *Jour. Exper. Med.*, 1922, 35, p. 391; M. Zuelzer, *Handbuch d. pathog. Protozoen*, 1925, II Lief., p. 1765); Encephalitis caused by *Encephali-*

* The important parasites are printed in boldface.

tozoon cuniculi (Levaditi, *Ann. de l'Inst. Pasteur*, 1924, 38, pp. 651–712; McCartney, *Jour. Exper. Med.*, 1924, 39, pp. 51–61); filterable virus (Andrewes and Miller, *Jour. Exper. Med.*, 1924, 40, pp. 789–796); tularemia (Shelton, *Jour. Am. Med. Assoc.*, 1925, 85, pp. 1019–1020).

Parasitic Invasions and Diseases

Ectoparasites:

Lice: **Haemodipsus ventricosus** (transmitter of tularemia).
Fleas: Pulex irritans, Ctenocephalus felis.

Parasites of mange and ear scabies:

Sarcoptes scabiei var. **cuniculi**, **Psoroptes communis** var. **cuniculi**; Chorioptes symbiotes var. cuniculi; Notoedres cuniculi.

Endoparasites:

Peritoneal cavity and omentum: **Cysticercus pisiformis.**

Intestinal tract:

Cestoda: **Cittotaenia pectinata**, **Cittotaenia denticulata**, Cittotaenia ctenoides, Andrya cuniculi, Anoplocephala wimerosa.
Nematoda: **Strongyloides papillosus** (Hall, pp. 7–9), **Trichuris leporis** (Hall, pp. 24–25) in large intestines, Trichinella spiralis (Hall, pp. 10–12); **Oxyuris ambigua** (Hall, pp. 68–69) in caecum and large intestines.
Protozoa: **Eimeria stiedae**, Obeliscus cuniculi (Graybill 1923) in stomach, Lamblia cuniculi; Chlamdophrys stercorea; Amoeba enterica.
Liver: **Eimeria stiedae**; Cysticercus pisiformis.
Lungs: Synthecaulus pulmonalis (Hall, pp. 163–167).

In connective tissue of subcutaneous of skeleton muscles: Coenurus serialis.
In any organ, tissue, or blood: Echinococcus granulosus; Trypanosoma cuniculi; Toxoplasma cuniculi; Spirochaeta raillieti.

Mice. White mice are usually employed in the technic of typing the various pneumococci and the toxin of *B. botulinus*. The animals must be kept in warm rooms, which are not too light. Jars or metal boxes with sawdust and cotton or 'excelsior' bedding serve as breeding cages. Cleanliness is very important in order to reduce the odor and to prevent the infestation of the animals with fleas. Mice mature in from 2 to 3 months; the gestation period is 16–21 days and the number of young varies from 4 to 6.

Feeding.—Whole milk, bread, and from time to time crushed barley or oats. It is advisable to have constantly available for the mice a supply of clean water furnished by a drinking fountain (test tube with small opening at bottom and closed by a rubber stopper).

Spontaneous infections.—Acute and chronic infections with spleen and liver lesions caused by *B. aertryke* and *B. enteritidis;* lung and kidney nodules caused by *B. pseudotuberculosis muris* (Kutscher); pneumonia caused by various microörganisms.

Parasitic Invasions and Diseases

Ectoparasites of gray and white mice:
Mites, lice and fleas: **Ceratophyllus fasciatus; Leptopsyllus musculi**; Ctenocephalus serraticeps.

Endoparasites: **Councilmania muris and decumani, Eimeria muris.**
Intestinal tract: **Hymenolepis murina** and diminuta; Oxyrhyuris obvelata (Hall, p. 81); Ollulanŭs triscuspis (Hall, p. 177); **Protospirra muuris** (Hall, p. 204); Pfeifferella schubergi.
Liver: Cysticercus fasciolaris.
Kidney: Klossiella muris: Eimeria muris.

For other parasites consult *The Rat and its Relation to Public Health,* pp. 115–116.

Rats. The albino rat is mainly used in research work. Care must be exercised in handling these animals, as the sharp incisor teeth are capable of inflicting a severe wound, and there is always a possibility of rat-bite fever ensuing. They should be held by the loose tissue at the nape of the neck with a pair of crucible tongs; the animal is kept taut by pulling on the tail.

Details concerning the breeding and the rearing of rats for research purposes will be found in the book by M. Y. Greenman and F. L. Duhring. The period of gestation is about 22 days; the number of the litter 5–7.

Feeding.—Rolled oats, wheat bread, milk, and fresh clean water are given.

Spontaneous infections.—Acute and chronic pneumonia, bronchiectasis, lung abscesses caused by *B. actinoides* (Jones); otitis media caused by influenza-like organisms; pseudotuberculosis, acute and chronic infections caused by *B. enteritidis* (Gartner) and *B. aertryke.* Streptothricosis and spirochaetosis and deficiency diseases (see McCollum, E. V., *The Newer Knowledge of Nutrition,* ed. 2, New York, Macmillan Company, 1922).

Parasitic Invasions and Diseases

Ectoparasites: Mites, lice, and fleas (Ceratophyllus fasciatus).

Endoparasites:
Protozoa: Councilmania decumani and muris, Endamoebia ratti (Kessel) in intestines.
Cestoda: Hymenolepis murina (intestines); Cysticercus fasciolaris (liver); Trichniella spiralis (muscles, artificial infection).

For other parasites, particularly those of wild rats, consult *The Rat and its Relation to Public Health,* pp. 111–122.

Pigeons and Barnyard Fowl. These birds are used for experimental purposes only. They should be housed in large, airy, light cages. As feed, grains, corn, potato peelings, green leaves and fresh clean water are offered.

Spontaneous infections.—Septicemia caused by *Pasteurella avisepticus* and *B. sanguinarum;* filterable virus of fowl plague; avian tuberculosis; birdpox.

Parasitic Invasions and Diseases

Ectoparasites: Mites, Dermanyssus gallinae, scaly legmite (Cnemidocoptes mutans); lice (Lipeurus heterographus (head), Menopon biseriatum (body), etc., on fowl; Lipeurus baculus and Goniocotes compar (on pigeons), fleas (Pulex avium), Echidnophaga gallinaceans, ticks (Argas miniatus).

For details consult Farmer's Bulletin, pp. 801–897, and U. S. Department of Agriculture, Circular, p. 213.

Endoparasites:

Protozoa: *Eimeria Pfeifferi;* Trichomonas columbae; Chilomastix and Lamblia, Balantidium viride.

Pigeon: *Choanotaenia infundibulum,* Davainea echinobothrida.

Cestoda: D. crassula, Bertiella delafondi.

Fowl: *Hymenolepis carioca,* Choanotaenia infundibulum, Davainea tetragona, D. echinobothrida, D. cesticillus, Hymenolepis cantaniana.

Nematoda: Spiroptera pectinifera (gizzard of fowl), *Heterakis columbae* (small intestines); *Heterakis perspicillum* and H. papillosa (intestines fowl), Trichosoma columbae (pigeon) and Tr. retusum (fowl); Syngamus trachealis (trachea of fowl), Echinorhynchus polymorphus (intestines of fowl).

Air Sacs: Mites: Cytoleichus nudus.

Blood: Trypanosoma columbae; Haemoproteus columbae, Proteosoma danielewskyi.

For further details consult Ward and Gallagher, pp. 178–225.

References:

Friedberger, E., and Schiff, F. Die Methoden des Tierversuchs *in* Kraus und Uhlenhuth's *Handbuch d. mikrobiologisch. Technik,* 1923, 2, p. 1563.

Greenmann, M. Y., and Duhring, F. L. *Breeding and Care of the Albino Rat for Research Purposes.* The Wistar Institute, Philadelphia, 1923.

Hall, M. C. Nematode parasites of mammals of the orders Rodentia, etc. *Proc.* U. S. Nat. Mus., 1916, 50, pp. 1–258.

Holman, W. L. Spontaneous infection in the guinea-pig. *Jour. Med. Res.,* 1916, 35, pp. 151–185.

HUTYRA, F., AND MARCK, J. *Spezielle Pathologie d. Haustiere.* 2 vols. Ed. 6, Jena, Gustav Fischer, 1922.

KESSEL, J. F. The distinguishing characteristics of the parasitic amoebae of culture rats and mice. *Univ. Calif. Publ. Zool.*, 1924, 20, p. 489.

REMLINGER, P., ET BEL, P. L'élévage du lapin et du cobaye dans les laboratoires. *Bull. de l'Inst. Pasteur,* 1924, 22, pp. 15–29.

STILES, CH. W., AND HASSALL, A. Compendium of animal parasites reported for rats and mice (genus mus) in *The Rat and its Relation to the Public Health.* U. S. Public Health and Marine Hospital Service, 1910.

WARD, A. R., AND GALLAGHER, B. A. *Diseases of Domesticated Birds.* New York, The Macmillan Company, 1920.

RULES REGARDING EXPERIMENTAL ANIMALS

The following University and Special Departmental rules must be followed strictly in order to insure humane treatment of the animals and to avoid dangers of infection:

General University Rules

1. Vagrant dogs and cats brought to this laboratory and purchased here shall be held at least as long as at the city pound, and shall be returned to their owners if claimed and identified.

2. Animals in the laboratory shall receive every consideration for their bodily comfort; they shall be kindly treated, properly fed, and their surroundings kept in the best possible sanitary condition.

3. No operations on animals shall be made except with the sanction of the director of the laboratory, who holds himself responsible for the importance of the problems studied and for the propriety of the procedures used in the solution of these problems.

4. In any operation likely to cause greater discomfort than that attending anesthetization, the animal shall first be rendered incapable of perceiving pain and shall be maintained in that condition until the operation is ended. Exceptions to this rule will be made by the director alone, and then only when anesthesia would defeat the object of the experiment. In such cases an anesthetic shall be used so far as possible and may be discontinued only so long as is absolutely essential for the necessary observations.

5. At the conclusion of the experiment the animal shall be killed painlessly. Exceptions to this rule will be made only when continuance of the animal's life is necessary to determine the result of the experiment. In that case, the same aseptic precautions shall be observed during the operation, and so far as possible the same care shall be taken to minimize discomforts during the convalescence as in a hospital for human beings.

By Order of the President of the University

Departmental Rules

1. Each animal, or set of animals, used by a student, or group of students, should be kept in a separate cage that has been carefully labeled (stamped tags) with the name of the owner and data concerning treatment, indicating particularly whether or not the treatment is of infectious nature. In the latter case, the caretaker should be instructed to disinfect the bedding with crude carbolic acid or similar disinfectant before cleaning the cage.

Animals not injected with living bacteria or those used for bleeding should be returned to the caretaker, who will place them among the 'second-hand' stock. The ear tags should not be removed. The final disposition of each animal must be noted on the original requisition blank and in the 'animal book.'

2. An inoculated animal should be placed by itself in a suitable cage or jar; when a number of animals are injected with the same material they may be placed in the same cage. Rabbits and guinea pigs should be numbered by using suitable ear tags or by tattooing with India ink; mice and even guinea pigs may be designated by painting the back, or flank, or the inside of the ears with anilin dyes.

3. *All experimental animals, whatever the cause of death, should be autopsied as a routine.*

ANIMAL INOCULATIONS

The use of living animals may become a necessary procedure for the following reasons:

1. Determination of pathogenic properties of bacteria already isolated in pure culture.
2. Isolation of pathogenic organisms from mixture.
3. Identification of pathogenic bacteria.
4. Study of the problems of infection and immunity.

The animals usually employed for these types of experiments are the guinea pig, rabbit, mouse, and rat; the commonest method of inoculation is by means of a hypodermic needle and syringe. It is important to consider briefly the various methods of inoculation, the equipment needed, the preparation of the material to be inoculated, and the preparation of the animal.

Apparatus Required for Animal Inoculations

Hypodermic syringes "Luer" all glass type 2 c.c. and 5 c.c. graduated in tenths; a selection of needles fine bore No. 25 gauge; medium bore for general use No. 21 or 22 gauge; large bore Nos. 16–18 gauge for thick suspensions; operating board or holding devices; scissors, needles and silk; all sterilized by the methods described on page 16; razor, liquid soap and pot of warm water, shaving brush, sterile gauze and cotton, 70 per cent alcohol, ether, iodine solution, collodium, adhesive plaster, disinfecting solution, saline solution, ear tags, labels and dyes for marking animals.

Care of Luer syringes.—If the syringe has been used for infectious materials, disinfect by rinsing in 2.5 per cent cresol solution, then boil from 5 to 15 minutes, depending upon the nature of material. After disinfecting wash in water until clean. If water fails to remove blood, etc., use the sulphuric acid-potassium bichromate mixture, then wash thoroughly in running water. Dry; place a well-fitting plunger and barrel separately in a large test tube containing a wad of gauze in the bottom; plug tubes with cotton covered with two layers of gauze; place in dry heat oven, sterilize at once. To prevent mistakes, never plug the tubes until just before placing them in the sterilizer. Sterilize for one hour at 170° to 180° C.

Care of hypodermic needles.—After using with infectious materials (exudates, blood, etc.), wash needle while on syringe with 2.5 per cent cresol solution by drawing in and discharging the solution several times; then boil in water. After using for injection of culture, disinfect needle at once by boiling. When disinfected, clean out the hub with a bit of cotton on forceps, and the lumen with fine wire. *Sharpen points frequently on soft oil stone,* and polish with jeweler's emery cloth, or 'Crocus cloth.' Dry lumen by passing first alcohol, then ether, through the needle. Place point downward in small test tube containing a plug of gauze in the bottom. Do not plug the opening of the tube until just before sterilizing. Sterilize with dry heat at 170° to 180° C. for one hour.

Preparation of Material Used for Inoculation

Fluid cultures are easily drawn through a medium bore needle; it will be found advantageous first to pour the culture into a small conical test tube or a small 2-inch Petri dish. Growth on solid media may be scraped off and emulsified in broth or saline, or the diluting

fluid may be poured over the culture slant or surface growth on plate which is then emulsified with a platinum needle. Toxins are prepared by filtration and manipulated in a similar manner as fluid media.

Pathological products as exudates, excretions, spinal fluid, etc., are treated as fluid cultivations. If the material is very thick or viscous, a small quantity of broth may be used to dilute it. Sputums are ground in a mortar and evenly emulsified before inoculation.

Tissues are divided into small pieces in a porcelain mortar by means of scissors. Some clean coarse sand, contained in a stoppered bottle, and sterilized by hot air, is then added to the mortar and the whole thoroughly ground with the pestle. When the tissue has been well ground up, saline or broth is slowly added and the mixture further triturated. On standing for a short time, the sand and the tissue settle to the bottom of the mortar and the supernatant fluid can be drawn into the syringe. When intravenous inoculation of tissue emulsion has to be employed, it is advisable either to strain the suspension through several layers of sterile gauze placed in a small funnel or to remove the coarse particles by centrifuging the suspension at low speed.

Preparation of the animal and methods of inoculation.—Before inoculation, the experimental animals should be carefully examined, to avoid the risk of employing any that are already diseased. The preliminary examination should comprise observation of the animal at rest and in motion; the appearance of the fur, feathers, inspection of the eyes, and of external orifices of the body; tactile examination of the body and limbs, palpation of the abdomen. The sex should be noted, the weight recorded, and the rectal temperature taken. The remaining items of importance are the time of inoculation, the material that is inoculated, and the method of inoculation.

Cutaneous inoculation (guinea pigs and rabbits).—Have the animal firmly held by an assistant or secured to the operating board; shave the area selected for inoculation; wash the denuded area with disinfectant, wash this off with alcohol and ether and allow the latter to evaporate. Scarify the skin by making short superficial incisions with the point of a sterile scalpel; rub the inoculum into the scarifications by means of a scalpel or inoculating needle; cover the inoculated area with sterile gauze held *in situ* by strips of adhesive plaster, or seal the edges with collodion.

Subcutaneous inoculation (guinea pigs on the abdomen or flank; rabbits on the abdomen, flank or back of the neck; mice and rats at the root of the tail or abdomen along median line).—An assistant holds the guinea pig or rabbit during the operation. The skin is shaven, washed, dried with alcohol and ether, and disinfected with tincture of

iodine. The operator picks up a fold of skin and introduces the point of the needle into the base of the fold, so that it lies in the subcutaneous tissue. At the moment the needle is withdrawn close the puncture with a wad of cotton soaked in cresol solution, to prevent the escape of any of the inoculum. Amounts up to 4 c.c. can be introduced into guinea pigs and 8 c.c. into rabbits. If a large amount is to be inoculated it is advisable to introduce small doses at several places.

Subcutaneous inoculations on mice are done as follows:

Either hold the mouse in a convenient metal holder or with the right hand hold the animal by the tail and then grasp the loose skin at the nape of the neck with the thumb and pointer finger of the left hand; turn the left hand over so that the mouse lies on its back and fix the tail by the little finger of the left hand. The manipulation will free the right hand for the cleaning of the area of inoculation, the handling of the syringe, and the inoculation. With a little practice the student can inoculate mice without the help of an assistant. Amounts up to 1 c.c. may be injected in mice.

If it is desired to inoculate pieces of tissue, the 'pocket method' is used. Remove from the material a small cube of 1 to 2 mm.; after shaving an appropriate area, disinfect the surface of the skin, cut an opening into the skin with scissors, open up the pocket with forceps, insert the tissue to bottom of the pocket with forceps, either suture the incision or seal it with collodion.

Intramuscular inoculation.—In the study of wound secretions containing anaerobic bacteria, guinea pigs are sometimes inoculated intramuscularly. The preparation is the same as for subcutaneous inoculation. An area over the large muscles of the hind legs is usually chosen. Steady the skin and thrust the needle boldly into the muscular tissue and inject the inoculum boldly. Sometimes glass pipettes are used for the inoculation; the tip is broken off after the material has been injected and is left in the muscle tissue.

Intraperitoneal inoculation.—Guinea pigs, rabbits, and mice are frequently injected in this manner. The animals are held and prepared in a similar manner as described for the subcutaneous inoculation. The skin is disinfected and painted with iodine. The assistant holds the animal with its head downward, so that the intestines fall toward the diaphragm. The skin is pinched up, the point of the needle passed into the subcutaneous tissue, and then downward through the abdominal wall into the peritoneal cavity. The needle puncture is covered with iodinized collodion. The risk of damage to the intestines is practically nil. Guinea pigs can safely receive 5 c.c., rabbits 10 c.c., and mice 2 c.c., of the inoculum. In the latter animal

the injections are made to one side of the middle line in the lower half of the abdomen.

Intracerebral inoculation.—The method is used in connection with the diagnostic study of rabies and herpes.

The head of the rabbit is shaved and sterilized with tincture of iodine. The animal is then anaesthetized with ether, and an incision is made in the skin midway between one eye and the ridge of the middle line at the junction of the parietal and occipital bones. The periosteum is deflected from the skull. With a small trephine a circular section of bone is removed and the material injected below the dura or into the brain substance, using a short (¼ inch) needle attached to a 1 c.c. syringe. The skin flap is turned back, seamed with one or two silk sutures, and the wound dressed with sterile gauze held in position with collodion. The amount to be inoculated should not exceed 0.25 c.c. of fluid.

Intravenous inoculation.—The method is usually practiced on the auricular vein of the rabbit. A great deal of skill is necessary to inject guinea pigs through the ear or jugular vein and mice by way of the tail veins.

The rabbit may be held by an assistant or is placed in a special box so that only its head protrudes. The hair over the vein should be dry shaved with a sharp razor. The vein may be distended for ease of inoculation either by vigorous rubbing with a piece of cotton or by holding the ear over an electric light globe, when the heat causes a dilation of the blood vessels. Xylol produces an inflammatory reaction and thrombosis of the veins and should not be used. A 22-gauge needle is most suitable for intravenous injections. The operator faces the animal holding the ear horizontal by means of the left hand. The needle is kept as parallel as possible to the marginal vein and the point inserted toward the head of the animal. When the injection is completed, the needle is withdrawn, and a small pellet of cotton placed on the vein, which is then compressed between the thumb and finger.

Feeding.—The inoculum is either applied on the tongue of the animal with the aid of an eye dropper, or small pieces of sterilized bread are soaked in the fluid inoculum and offered to the animals in a sterile capsule or Petri dish. Sometimes the feeding is done with the aid of a stomach tube.

Reference:

Roth, G. B. Intravenous administration to mice, rats and guinea pigs. *Public Health Reports;* Reprint No. 647, 1921, 36, p. 661.

OBSERVATIONS OF EXPERIMENTAL INFECTIONS DURING LIFE

Immediately upon the inoculation of an animal a period of clinical observation begins which should terminate only with the death of the animal.

The General Observations should take cognizance of:

(*a*) General appearance. Daily inspection should be given with a view to detecting symptoms, also to prevent any intercurrent infection.

(*b*) The *weight* should be recorded each day.

All experimental animals should be weighed before infection and every day or two afterward. Each weighing should be done, as nearly as possible, under the same conditions as the first one. The animal should have plenty of food and water before weighing. In warm weather water is imperative. A difference of 200 to 400 grams in rabbits betwen two weighings may be due entirely to the withholding of water. By observing these precautions the results of weighing become of significance. Weight is the best index of the physical well-being of the animals. A steady, slight decrease in weight is a sure indication of a chronic, wasting disease.

(*c*) The *temperature* of the animal should be taken in many experiments before beginning the particular experiment and subsequently on each successive day at the same hour.

The ordinary clinical thermometer, previously covered with vaseline, should be inserted well up into the rectum.

The accompanying table of normal averages may be of use in preventing the erroneous assumption that pyrexia is present in an animal which shows merely its own normal temperature.

The Special Observations comprise some or all of the following:

(*a*) The *site of inoculation* should be examined at, at least, weekly intervals.

(*b*) Any *local reaction* at the site of inoculation and any other readily accessible lesion should be carefully investigated. The secretions should be studied bacteriologically.

(*c*) The *peripheral blood* should be examined from time to time.

Haematological information as to the course and progress of an infection can be obtained from a bacteriological and a serological examination of the blood.

(1) *Haematological study.*—The number of red and white blood corpuscles are counted with the aid of a haemacytometer (Levy

chamber with Neubauer's ruling). The use of the apparatus is described in Stitt, E. R., *Practical Bacteriology, Blood Work and Animal Parasitology* (ed. 7; Philadelphia, Blakiston's Son and Co., 1923), pp. 292–297.

For a differential count or for the study of blood parasites the preparation of dried films is very important. Smears on slides are very satisfactory if made in the following manner:

A small drop of blood obtained from the veins of the ear (rabbit, guinea pig, and cat) or the tip of the tail (rat and mouse) or the axillary vein (pigeon and fowl) is placed on a clean slide. Touch the end of a clean slide, to be used as a spreader to the drop. Holding this slide at an angle of 45°, bring the drop into contact with a horizontal slide near one end, and, as soon as the blood runs out along the line of contact, advance the spread slide with an even sweep to the other end. The blood is pulled or drawn behind the edge of the advancing slide. An angle less than 45° makes a thinner film; one greater, a thicker film. Allow the film to dry in the air; fix with methyl alcohol and stain with Wright's polychrome methylene blue (modification of *Balch,* see Stitt, p. 303).

The *thick film* method is of great assistance in searching for bacteria, protozoa, and spirochaetes. A small drop of blood is smeared out over an area of the slide about equal to that of a small square cover glass and allowed to dry completely. Immerse and gently move the slide in a glass containing a 2 per cent solution of formalin to which has been added 1 per cent of glacial acetic acid. After laking is complete, treat the slide in the same way in a glass of tap water to remove all traces of acid; then wash very gently in distilled water and stain with dilute Giemsa solution (1 to 40) for 60 minutes. Wash in water and allow to dry with blotting or heat.

TABLE 3

NORMAL TEMPERATURE, PULSE AND RESPIRATION OF EXPERIMENTAL ANIMALS

Animal	Rectal temperature °C.	Pulse rate	Respiration per minute
Guinea Pig	38.6[2] (Minimum 37.8, maximum 40.5°)	150	100—150
Rabbit	39.6° (Minimum 38.3, maximum 40.8°) No temperature under 40.0° should be considered pathological.	120—140	50—60
Rat	37.9	—	210
Mouse	37.4	120	—
Cat	38.0—39.0	110—130	20—30
Pigeon	41.0—43.0	150—200	16—36
Fowl	40.5—42.0		12—28

TABLE 4

Haematology of Experimental Animals

Totals			Percentages					Remarks
Animal	Red cells	White cells	Lymphocytes	Large mono-nuclear	Polymorph. nuclear	Eosinophiles	Mast cells	
Guinea Pig	5,270,000	10–15,000	47.5	10.0	31.5	10.0	0.37	Polychromasia; basophilia and nucleated cells frequent; mono-nuclears contain often Kurlow's bodies.
Rabbit	5,250,000	7–14,000 (average 8150)	40.0	10.0	47.0	1.0	2.0	Nucleated red cells common; eosinophilous granules are coarse.
Mouse	9,725,000	7,400	37.0	15.0	46.0	1.25	0.75	Granules of polymorphonuclear leucocytes are usually poorly stained.
Rat	9,300,000	15,200	53.5	24.5	16.5	3.5	2.0	Nucleus of eosinophile cells is often ring-shaped.
Pigeon	4,055,500	10–31,000	42.0	13.5	41.0	1.5	2.0	Blood platelets 9,000–63,000; red cells are oval nucleated discs.
Fowl	3,117,000	35–60,000	51.5	12.3	29.5	4.5	2.2	Red cells are oval nucleated discs; granules of polymorphonuclear cells are rod-shaped.

Some notes on the normal character of the blood of the commoner laboratory animals are here presented for reference.

(2) *Bacteriological examination* is mainly directed to the demonstration of the presence in the circulating blood of the organisms injected into the animal. For this purpose several cubic centimeters of blood are taken in a glass syringe, either from an accessible vein or by cardiac puncture under aseptic precautions.

(3) The *serological examination* of the blood is directed to the demonstration of the presence of certain specific antibodies in the sera of experimentally infected animals and within certain limits to an estimation of their amounts. The methods of collecting blood are as follows:

(A) Dry shave the skin over the course of the posterior auricular vein. Cleanse and disinfect the ear with alcohol and ether; puncture the vein with a needle or a sharp scalpel. The blood is either aspirated into a Pasteur pipette or allowed to drop into a test tube. This method works satisfactorily on rabbits and guinea pigs. The bleeding is stopped with the aid of a small clamp or with cotton soaked in a solution of iron sergin chloride.

(B) Large amounts of blood are usually secured by cardiac puncture on carotid bleeding. The methods will be demonstrated; details can be found in Kolmer, J., *A Practical Textbook of Infection, Immunity and Biologic Therapy*, ed. 3; Philadelphia, W. B. Saunders Company, 1923, pp. 33–41.

The important serological methods are described in the laboratory exercises.

References:

BURNETT, S. H. *The Clinical Pathology of the Blood of Domesticated Animals.* Ithaca, 1908.

KLIENEBERGER, C., AND CARL, W. *Die Blutmorphologie der Laboratoriums-Tiere.* Leipzig, Johann Ambrosius Barth, 1912.

MAREK, JOSEF. *Lehrbuch d. klinischen Diagnostik d. inneren Krankheiten d. Haustiere* (Jena, Gustav Fischer, 1912), pp. 150–151.

TIGERSTEDT. *In* Winternitz's *Handbuch d. vergleich. Physiol.* (Jena, Gustav Fischer, 1911–14, 3, 2ter Teil), pp. 58–60.

ANIMAL AUTOPSY

All experimental animals should be autopsied as a routine. The purpose of a complete autopsy is threefold:

(1) To recover the organisms previously injected.

(2) To determine the lesions produced by the organisms.

(3) To establish the nature and duration of the disease, its anatomical distribution, and the cause of death.

No branch of medicine demands sounder knowledge, a more accurate and systematic technic, keener powers of observation, discrimination, and interpretation, than post-mortem pathology. Since the fundamentals of every medical activity rest ultimately on the anatomical or physiologic anatomical recognition of disease processes it is most desirable that every opportunity offered in the practical course of bacteriology be taken to become acquainted with the usual lesions which characterize a degeneration, inflammation, circulatory disturbance, or a malformation. Regular systematic autopsies on laboratory animals may help in preparing the student for his subsequent studies in human pathology. With these points in view the outline given below is perhaps more extensive than found in most guides to bacteriological technic, but experience has shown that a great deal of interest in this important subject can be created by systematic post-mortem examinations.

In performing an autopsy a few general rules should be observed:

(1) The student should be familiar with the normal anatomy of the animal to be necropsied. A few diagrams showing the location and shape of the internal organs of the rabbit, guinea pig, and mouse are available in the laboratory. The average weight of the normal organs is given below. For further details the student should consult the following publications:

Bensley, B. A. *Practical Anatomy of the Rabbit.* Ed. 3; Philadelphia, P. Blakiston's Son and Co., 1921.

Donaldson, H. H. *The Rat. Data and Reference Tables.* Ed. 2; Philadelphia, The Wistar Institute of Anatomy, 1924.

Hunt, H. R. *A Laboratory Manual of the Anatomy of the Rat.* New York, the Macmillan Company, 1924.

Martin, P. *Lehrbuch der Anatomie des Haussäugetiere* (*Anatomie der Impfsäugetiere*). Ed. 2; Stuttgart, Schickhardt and Ebner, Ed. 4, Lief. 3, pp. 303–408.

(2) The instruments which are needed for the performance of an autopsy should be in good condition and repeatedly sharpened.

(3) For many reasons it is important to carry out the post-mortem examination as soon after death as possible.

(4) Whenever possible the examination should be conducted in a good light. Artificial light is undesirable, and its effects upon colors are very misleading; for example, jaundice is very easily overlooked.

(5) When the primary reason for the autopsy is to recover organisms previously injected into the animal, the examination must be conducted with strict aseptic precautions.

(6) Great care must be exercised when the animal is infected with highly pathogenic bacteria, otherwise the infection may be disseminated with danger to the operator and other workers. Rubber gloves should be worn.

(7) A cadaver should be examined systematically; examine the exterior, then the interior, abdomen, thorax, pelvic cavity, finally the bones, joints and head. Never displace an organ from its location before its relation to the surrounding has been established.

(8) Every organ must be removed from the body and carefully examined with regard to size, shape, color, consistency, and internal structure.

(9) Every autopsy must be recorded in the daybook. The student should learn to present a careful *description* of the findings. Notes written at the time of the autopsy are much more accurate than those written afterwards from memory, when the opportunity of filling in gaps or confirming details has passed.

(10) The dissected carcasses must be either destroyed by immersion in concentrated sulphuric acid (mice and rats) or by incineration.

Materials, Instruments, and Preparations Required

Suitable *animal boards*, on which the carcasses can be fixed in the supine position. Mice and rats are fastened with pins to wooden board covered by a layer of linoleum. The boards are placed on sterilizable enamel or aluminum trays.

Instruments.—Four scalpels; four pairs of ordinary scissors; six pairs of mouse-toothed forceps; small bone forceps for the opening of the skull; a searing iron—a soldering iron is excellent for searing purposes; sterile capillary pipettes; sterile Petri dishes with a layer of sterile filter paper; sterile test tubes; a dish with liquid petrolatum held at 180 ° C., and tubes of culture media.

The knives are sterilized in concentrated liquor cresolis, and the metal instruments by boiling in the usual sterilizing bath. When ready for use, the tray of instruments is lifted out of the sterilizer and laid on a tray which is covered by a spread towel previously soaked in 1:1000 solution of bichloride of mercury.

When complete cultures have to be made, immerse the animal completely in 5 per cent liquor cresolis for 10 minutes. This destroys most of the surface organisms, and prevents the dust in the fur from getting into the air and causing contaminations. When the animal is fixed to the board, towels moistened with antiseptics are placed over the head and lower extremities.

Outline of a Complete Autopsy

(1) Before the autopsy of an animal is begun, a very careful *external examination* is made. The following points should be noted: weight, signs of death, cooling of the cadaver, rigor mortis, signs of decomposition, the visible mucous membranes, natural openings, eyes, skin and ectoparasites, wounds whether inflicted during life or after death.

(2) Arrange the instruments, the culture dishes, and the searing iron; immerse the body of the animal in the antiseptic and then fasten it to the board. Take up a position on the right side of the body, keep the instruments on the right side of the board and begin the *internal examination.*

(3) An incision is made through the skin along the median line from the tip of the jaw to the anus; the skin is widely dissected exposing the abdominal and chest muscles; the forelegs are freed from the thorax. Place the now infected instrument in a tray with cresol solution.

(4) Inspect the *seat of inoculation* or in general the subcutaneous tissue and the mammary glands. Remove material, pus, or other exudation in pipettes for subsequent examination. Make films on slide if necessary. Inspect and then dissect the inguinal and axillary lymph-nodes and place in sterile Petri dishes.

(5) Sear a broad track in the middle line of the abdominal wall and the whole of the exposed surface of the *thorax.* Using another set of instruments open the peritoneal cavity by an incision in the center of the seared line. Deflect the abdominal wall to each side. Observe the condition of the omentum, the mesentery, the viscera, and the peritoneal surface of the intestines.

(6) Remove in a capillary pipette specimens of peritoneal fluid for cultures and smears. If necessary make impression preparations from the liver surface.

(7) *Pleural cavity.* The ensiform cartilage is now tightly gripped with a pair of strong forceps, and by means of a sterile pair of scissors a cut is made in the diaphragm and on each side of the chest through the costal cartilages. The sternum is raised and pulled toward the head. Place the infected instruments in the cresol solution.

(8) Observe the condition of the thymus, the mediastinal lymph-nodes, and the lungs. Collect a sample of pleuritic effusion, if such is present.

(9) Raise the pericardial sac with a fresh pair of forceps and burn through this structure with a searing iron. Collect a sample of pericardial fluid for cultural and microscopical examination.

(10) Sear the surface of the right ventricle and pass a sterile capillary pipette furnished with a teat through the heart wall. Blood can thus be withdrawn and inoculated into various media. Collect a further sample of blood for subsequent investigation as to the presence of antibodies.

(11) Using fresh instruments, remove the lungs, heart, and lymph-nodes by cutting the organs free at the hilum. Care must be taken not to open the oesophagus. Place the organs in a sterile Petri dish.

(12) Resume the examination of the *peritoneal cavity.* Collect a specimen of urine from the distended bladder in a large pipette for further examination. In a similar manner collect a specimen of bile from the gall bladder.

(13) Excise with separate instruments the spleen, liver, kidneys, mesenteric, hepatic, lumbar and para-aortic lymph-nodes, uterus, ovaries, seminal vesicles, and testes and place them in sterile Petri dishes.

(14) Examine the intestinal canal, open the duodenum, the caecum and colon, remove specimens of the contents and parasites, if present, for further examination. In the rabbit examine in particular the sacculus rotundatus and the caecum.

(15) Dissect out a long bone from one upper and lower limb and one of the largest ribs. Break the shaft of the bone with a pair of bone forceps and remove with separate instruments specimens of bone marrow for culture and film preparations.

(16) Inspect the muscles of the limbs, make cultures and smears from hemorrhagic areas or exudations.

(17) Examine the joints, sear the surface with the iron, and push a capillary pipette into the joint cavity. Collect synovial fluid or pus for cultures and microscopical examination.

(18) Examine the neck submaxillary gland; dissect the tongue, and the larynx, trachea, and oesophagus. Inspect the posterior nasal passages, gums, and teeth. Collect specimens for cultures if necessary.

(19) In some instances it may be necessary to examine the *cranial cavity* or to remove the *spinal cord.* Return the viscera to the abdominal cavity; draw the flaps of the skin together and cover the incision with cotton soaked in cresol solution. Reverse the animal and fix the limbs so that the body is now dorsum uppermost. This manipulation is done either with the aid of strong forceps or by hands covered with rubber gloves.

(20) Make a longitudinal incision in the middle line from snout to root of tail and four transverse incisions—one joining the roots of the two ears, one across the body at the level of the scapulas, another at the level of the costal margin, and the last across the upper level of the pelvis. Dissect out the muscles lying in the furrow on each side of the spinal processes. Cut through the bases of the transverse processes with bone forceps. Cut away the vault of the skull. Cut through the roots of the nerves, examine and then remove the brain and spinal cord; place in a large dish for examination. Prepare films and cultures if necessary. The removal of the brain and cord is a tedious process and it is difficult to avoid injury to these structures.

(21) Having completed the dissection, the examination and the removal of the organs, gather up the infected instruments, return the knives to the cresol solution and the metal instruments to the sterilizer and disinfect by boiling for 15 minutes. While working with anaerobes heat the instruments in liquid petrolatum for 10 minutes at 180° C.

(22) Remove the cadaver from the board and immerse in crude sulphuric acid or wrap it in strong paper and place it in a pail provided with a lid ready for transportation to the crematorium. The board is disinfected with cresol solution.

(23) When the animal is infected with highly pathogenic organisms, the worker must wear rubber gloves. The animal is soaked in antiseptic solution as before, and nailed to a rough piece of board of appropriate size. The board is then placed in a large enameled tray. The autopsy is carefully performed in the usual way. The whole carcass is then covered with 20 per cent lysol. The whole contents of the tray—board and carcass—are then destroyed in the incinerator. The rubber gloves, instruments, and tray are thoroughly sterilized.

(24) The various organs and tissues removed at autopsy are now examined and cultured. In case an organ has been soiled with intestinal content it is first examined, pieces are removed for histologic study and a portion is then dipped for 5 seconds in paraffin oil having a temperature of 180° C. Divide the organ with a pair of scissors and dab the cut surface on to a surface plate or remove with a loop particles of tissue and transfer to fluid mediums.

Technic and Description of Organs

The description of an organ whether mental or written should consist of a systematic routine in which some or all of the following points are considered: position, size, weight, shape, color, odor, consistency, incision, incision fluid, cut surface, and contents. One should avoid the use of the words *normal* and *unaltered* as they create a disposition to slight the work and make one a poorer observer. As a guide for the descriptions use MacCallum, *A Textbook of Pathology.*

Examine first the structures of the *pleural cavity.* Note appearance of the mediastinum (inflammation, enlarged lymph-nodes), pleural lining (pleurisy, adhesions, ecchymoses, empyema), pericardium (serous effusion, hemorrhages, fibrinous, purulent pericarditis), and the heart muscle (atrophy, hypertrophy, fatty changes, infarctions, hemorrhages, and milky scars). Separate the heart from the lungs. Open the cavities of the *heart* as follows:

Incise the anterior wall of the right ventricle with a pair of scissors. Keep to the right of the septum and begin the incision midway between the base and apex of the ventricle, cutting downward toward the apex. A blade of the long scissors should now be introduced into the incision and passed up close to the anterior wall and ventricular septum of the heart until it emerges from the cut end of the pulmonary artery. Keep well toward the left margin of the pulmonary artery and cut upward, taking care to pass between two of the pulmonary valve segments. The method of opening the left ventricle is similar to that adopted for the right. The long blade of the scissors is this time brought out of the aortic orifice, which also should be incised between its valve segments. Inspect the blood clots if present; note the appearance of the myocardium in sections; examine the valves (endocarditis).

Place the *lungs* with the trachea toward the autopsist. Examine the bronchial lymph-nodes. Insert a blade of a pair of scissors in the trachea and cut through the dorsal surface to the bronchi. Lay open the bronchi in the same manner and inspect (tracheitis, bronchitis parasites). Note any changes on the pleura (fibrinous pleuritis,

adhesions). Palpate the lung thoroughly for areas of consolidation, nodules, etc. Cut the lung into sections by making longitudinal incisions. Scrape the surface of the cut sections and if foam can be collected upon the blade, lung oedema is indicated. When the lung presents a deep red or gray consistency a very small piece of it should be excised and placed in a glass with water (+ bichloride 1:1000). Note the various changes which may be present (emphysema, hypostatic congestion, oedema, atelectasis, lobar and bronchopneumonia, infarction, embolic abscess, gangrene, tuberculous and parasitic nodules). Inspect the teeth, jaws, tongue, larynx, pharynx, and upper food passage (abscesses, ulcers, various types of inflammation).

A systematic inspection of the abdominal contents is now carried out.

The *peritoneum* is examined (peritonitis, hemorrhagic effusion). The weight (rabbit 0.1–0.3 per cent of body weight; guinea pig 2.5×3.0 cm. long, rat 3.5×4.5 cm. long, mouse $1.5 \times 0.3 \times 0.2$), shape, and consistency of the *spleen* is determined. On cut section the pulp, malpighian corpuscles, trabeculae, and capsule are examined (enlargement, atrophy, congestion, infarcation, abscesses).

The *liver* is laid before the operator with the anterior or convex side upward. The thickness is estimated and the organ is cut in thin sections; the capsule is stripped and the consistency is determined by pressing the parenchyma with a knife blade. When degenerated the consistency is friable, usually the edges are rounded. Note the various changes which may occur (perihepatitis, fatty and cloudy swelling, congestion, cirrhosis, abscesses, infarcts, tuberculous, parasitic nodules, cholangitis caused by Eimeria). Never fail to examine the *gall bladder* in the rabbit, guinea pig, mouse, and the birds (cholecystitis with sand-like débris).

Hold the *kidneys* with a pair of forceps and make a smooth incision from the convex margin through the parenchyma to the hilus, severing the kidney in halves so that it may be laid open like a book. Examine the capsule, the cortex, and the renal pelvis (congestion, acute and chronic nephritis, infarction, abscesses, pyelitis, cysts, hydronephorosis). Section the *adrenals* and note the appearance of the cortex (hypertrophy, hemorrhages).

Incise the *stomach* at the larger curvature; occasionally in the rabbit post-mortem digestion suggests a rupture of the stomach; examine the mucosa for hemorrhages, ulcers, or erosions. The *intestinal canal* is opened by incisions along the insertion of the mesentery in order to expose the lymph follicles. The content, the appearance of the mucous membrane, and the lymph tissues of the caecum and sacculus rotundatus deserve attention (catarrhal or hemorrhagic enteritis, parasites). Dislocations of the intestines in the form of

post-mortem intussusception are observed in the rabbits and guinea pigs.

If necessary expose the *uterus* and *ovaries* and inspect the mucous membrane (metritis, abscesses in the wall, mumified foetuses, etc.). The *male genital organs* are sometimes the seat of lesions (exudates in the spermatic vesicles; suppurative periorchitis, atrophy, etc.).

The *brain* is placed with the dorsal side upward, the medulla oblongata extending toward the autopsist. Examine the meninges (hyperaemia, oedema, meningitis caused by middle ear infections). Spread the hemispheres of the cerebrum slightly apart and with a sharp scalpel make a longitudinal incision in the inner side of each hemisphere. Lay open the lateral ventricles and expose the caudate nucleus and hippocampus. Make transverse sections through the cerebrum and cerebellum if necessary. *Specimens* for diagnosis for rabies are removed from the hippocampus and cerebellum.

On the exposed *long bones* note the appearance of the periosteum, the color and consistency of the marrow, and condition of the shaft (scurvy, osteomyelitis, necrosis). The shoulder, the knee, and the tarso- and metatarso-phalangeal joints are opened by transverse incisions, and the content, the synovial membrane, the articular cartilages, and condition of the epiphyseal parts of the bones are carefully scrutinized (purulent arthritis, hemorrhagic effusion, etc.).

The *muscles* are exposed by special incisions and carefully examined for hemorrhages, effusions, parasites, etc.

The autopsy report is summarized in the form of a pathologic-anatomical diagnosis in which the findings in technical terms are arranged in accordance with their importance. For example:

Septicemia with lobar pneumonia, fibrinous pleurisy, splenic tumor, cardiac dilatation, parenchymatous changes in the liver and kidney, Trichuris leporis and coccidiosis of the liver.

HISTOLOGICAL EXAMINATION OF TISSUES

Having completed the preparation of cultures remove thin slices (about ⅛ inch thick) of various organs at leisure and place each in separate containers of fixing fluid for future sectioning. Always use plenty of fixing fluid. The following *fixatives* are recommended for ordinary bacteriological investigations of tissues: formalin, corrosive sublimate-acetic acid solution.

Formalin fixation.—Ten per cent commercial formalin (40 per cent solution of formaldehyde free from paraldehyde) in normal salt solution at a temperature of 30–36° C. is a good fixative and permits the use of most of the bacterial stains. Fixation and hardening is usually

complete in from 6 to 48 hours. The specimens *must be* washed in running water for several hours, then transferred to 50 per cent, and then 70 per cent, alcohol. They should be imbedded, not kept, in the latter fluid.

Corrosive sublimate-acetic acid.—To saturated solution of bichloride of mercury in distilled water warmed to 30° C., add shortly before use 5 per cent glacial acetic acid. Fix for from 4 to 48 hours. Rinse in water and transfer to 50 and then 70 per cent alcohol. This fixative gives excellent results with most of the bacterial stains provided the bichloride is carefully removed from the sections and not before the specimens are imbedded.

After fixation by either method and transference to 70 per cent alcohol, carefully cut pieces of tissues are imbedded in paraffin.

Imbedding and Section Cutting

(1) Dehydrate tissues in 90 per cent alcohol for 24 hours.

(2) Transfer to 95 per cent alcohol for 24 hours.

(3) Complete dehydration in two changes of absolute alcohol held in a glass-stoppered dish with anhydrous copper sulfate for from 4 to 12 hours or longer depending on the size of the specimen.

(4) Transfer to xylol for 1 hour.

(5) Place in fresh xylol for 2 hours.

(6) Transfer tissue for 2 hours to a mixture of paraffin wax and xylol (2 to 1) which is held in a closed dish on top of the paraffin oven.

(7) Place in pure melted paraffin in the oven at 52–56° C. for 2 hours.

(8) Transfer twice to fresh paraffin for from 1 to 2 hours.

(9) Imbed the tissue in blocks of paraffin (melting point 52–54° C.).

The periods of time in the various fluids must be prolonged when large pieces of tissues are employed. The specimens may be imbedded in paper molds or in pans. The inside of the pan must be coated with a thin layer of glycerin; this keeps the paraffin from sticking. The pan containing the arranged tissues is floated on ice-cold water. This insures rapid solidification of the paraffin without crystallization and gives better cutting qualities to the blocks. These are cut out, trimmed with a knife, and sections of 5μ thick cut by means of a microtome. The sections are flattened on warm water, floated on albumenized (filtered egg albumin and glycerin equal parts of each, preserved by the addition of 1 per cent sodium salicylate) slides and allowed to dry in the incubator.

Staining of Paraffin Sections

Four methods found suitable for the demonstration of microorganisms in tissues are given. For additional details on staining of tissues for histologic studies the student is referred to the works on histology or pathologic technic. Use Coplin jars for reagents except absolute alcohol, which should be kept in glass-stoppered containers. The sections of specimens fixed in formalin are treated as follows:

(1) Remove paraffin from sections with xylol, 5 minutes.

(2) Remove xylol with absolute alcohol and 95 per cent alcohol, 5 minutes for each.

(3) Run down to water through the following grades of alcohol, allowing alcohol about 5 minutes in each alcohol, 90–80–70–50.

(4) Place in distilled water.

(5) Stain, differentiate, dehydrate and mount in cedar oil.

The sections *fixed in corrosive of sublimate must be treated as follows:*

(1) Remove paraffin from section with xylol, 5 minutes.

(2) Remove xylol with absolute alcohol and 95 per cent alcohol.

(3) Transfer to 70 per cent alcohol tinged with Lugol's solution to a light brown color 15 to 30 minutes.

(4) Place in 95 per cent alcohol to remove iodine.

(5) Pass through 70 and 50 per cent alcohol to distilled water.

(6) Place in 0.5 per cent aqueous solution of sodium hyposulfite, 5–10 minutes.

(7) Wash in running water.

(8) Transfer to distilled water, stain, differentiate, dehydrate and mount according to the staining method chosen.

Simple stains.—Transfer the mounted sections from distilled water to an aqueous solution of old Manson's blue (1:20) and stain for 20–60 minutes. Wash rapidly in 1:1000 acetic acid solution; dehydrate with absolute alcohol 20 seconds, clear with xylol; mount in cedar oil.

Gram stain (method recommended by Pappenheimer).—Stain in Stirling's gentian-violet 5 minutes; rinse in water; then cover with Gram's iodine 1–2 minutes; blot gently with filter paper. Decolorize with aniline oil-xylol (āā) to a pale violet color. Rinse in absolute alcohol; transfer to distilled water; counterstain with aqueous safranin (½ per cent) 30 seconds. Rinse in distilled water; blot with filter

paper; pass rapidly through absolute alcohol to xylol; mount in neutral balsam or cedar oil.

Stain for tubercle and lepra bacilli (Konrich's method).—Transfer sections to a concentrated solution of Ziehl-Neelsen's carbolfuchsin which is held in a glass stoppered container. Stain for 24 hours at 37° C.; rinse carefully and repeatedly in distilled water; differentiate in a fresh 10 per cent aqueous solution of sodium sulfite until nearly colorless; rinse in water; counterstain for 1 to 2 minutes with an aqueous solution of malchite green (saturated aqueous solution 50 and distilled water 100); rinse in distilled water; pass rapidly through 95 per cent absolute alcohol to xylol and mount in cedar oil.

Reference:

KONRICH. Eine neue Färbung für Tuberkelbazillen. *Deutsch. Med. Wochnschr.*, 1920, 46, p. 741.

Polychromatic Giemsa stain (Wolbach's modification).—Beautiful sections are obtained after fixation with corrosive sublimate.

Transfer the sections from distilled water to the following solutions:

Distilled water	100 c.c.
Giemsa solution	60 drops
Methyl alcohol	10 c.c.
0.5 per cent aqueous sodium carbonate	2 drops

Stain for 24 hours; renew the staining solution twice. Rinse in distilled water and differentiate as follows:

(1) Fifteen per cent colophonium in acetone a few seconds.

(2) Transfer to the same reagent for a few seconds.

(3) Place in a mixture of acetone 70 parts and xylol 30 parts.

(4) Transfer to a mixture of equal parts of acetone and xylol.

(5) Clear in xylol and mount in neutral cedar oil, *never* in balsam. Control the progress of differentiation under the microscope.

For special staining methods consult:

JOANNOVIC, G. Methoden d. Färbung von Microorganismen *in* Schnitt, Kraus und Uhlenhuth's *Handbuch der Microbiologischen Technik.* Berlin, Urgan and Schwarzenberg, 1923, 1, pp. 413–435.

MALLORY, F. B., AND WRIGHT, J. H. *Pathological Technique.* Ed. 7; Philadelphia and London, W. B. Saunders Co., 1918.

MCJUNKIN, F. A. *Clinical Microscopy and Chemistry.* Philadelphia and London, W. B. Saunders Co., 1919, pp. 317–381.

PART II

WEEKLY EXERCISES IN PRACTICAL BACTERIOLOGY

PREPARED BY

P. SCHOENHOLZ

PART II

WEEKLY EXERCISES IN PRACTICAL BACTERIOLOGY

PREPARED BY

P. SCHOENHOLZ

The laboratory work is so arranged that the student may execute the exercises without much assistance and advice. It is recommended that he read the weekly program very carefully, assemble the equipment and seek advice before he begins the exercises. For each day a definite program is prescribed. In the plan adopted the special materials required to carry out the work are outlined for each week. Ordinary desk equipment, test tubes, Petri dishes, etc., are not enumerated unless an extra large supply are required; it is assumed that such apparatus is always available. All glassware, etc., used in an exercise should be sterile unless otherwise stated.

It has been noted in the past that students are, at times, apt to be careless in handling and disposing of cultures, on the assumption that such carelessness is justifiable when dealing with non-pathogenic organisms. Such technic will not be tolerated by the department. A medium once inoculated with a living organism is contaminated—whether it shows growth subsequent to incubation or not. (The optimum conditions for growth may not have been chosen.) Such inoculated cultures and all specimens must always be properly sterilized before the containers are emptied and cleaned. Facilities for the proper sterilization and washing of glassware are provided. All contaminated material is carefully placed in buckets provided for that purpose. Test tubes, flasks, etc., should be placed in the receptacle in an upright position so that the contents do not flow out. All containers must be properly stoppered. *Never* leave loose test tubes on the table. If the buckets are filled the test tubes should be placed in baskets and left for sterilization.

The course extends over a period of 16 weeks with four afternoon laboratory periods. The lectures are given on the second and third day; the time-consuming exercises are therefore planned for the first and fourth day. It is most unfortunate that the present medical cur-

riculum does not permit of the organization of a bacteriological course with daily laboratory periods. Continuous observation of the cultures, animals, etc., is most essential in practical laboratory work. The student will profit a great deal more from the work outlined in the exercises if he arranges his work so that he can examine his cultures, specimens, etc., daily instead of merely during the assigned periods. The department is open daily from 8 A.M. to 6:30 P.M. and the students are invited to spend their spare hours in the laboratory or in the library.

The questions which are added to some of the exercises must be answered in the daybook.

WEEKLY EXERCISES IN PRACTICAL BACTERIOLOGY

First Week

I. Preparation of glassware.
II. Preparation of stains.
III. Examination of young and old cultures of *B. prodigiosus* and *Staphylococcus albus.*
IV. Examination of pure culture of *B. subtilis.*
V. Examination of organic infusion.
VI. Qualitative examination of aerobic bacteria in the air.

Special Materials Required—

Sterile Petri dishes.
Agar "deeps" (No. 57).
Mixed young and old cultures of *B. prodigiosus* and *Staphylococcus albus* suspended in saline.
Organic infusion.
Chlorotone.

2d day.

I. Preparation of Glassware.

1. Cleaning of glassware—follow the methods outlined on page 11.

2. Preparation of glassware for sterilization—demonstration of plugging glassware.

3. Principles of handling dry air, Arnold and autoclave sterilizers.

II. Preparation of Stains. Briefly summarize in your notebook, the preparation and composition of the more important staining solutions. State the fundamental principles involved in staining bacteria.

3d day.

Demonstration of general bacteriologic technic.

III. Examine Culture of *B. prodigiosus* and *Staphylococcus albus.*

1. In hanging drop preparation (see p. 19) distinguish true motility from Brownian movement and movement due to currents.

2. Stained by Gram's method. Rods and cocci will be seen. Distinguish between Gram + and Gram — organisms. Is there any difference in the staining reactions of young and old cultures of staphylococci?

IV. Examine a Pure Culture of *B. subtilis* in the same manner. Are they motile? Are all of the organisms uniformly stained? Are any spores present? If so stain with Burke's spore stain. Do they all stain uniformly? If not why do they fail to do so?

Reference:

Koser and Mills. Differential staining of living and dead bacterial spores. *Jour. Bacteriol.*, 1925, 10, p. 25.

V. Examine separately the Scum and Sediment of an Organic Infusion.

1. In hanging drop preparation.
2. In films stained with:
   (*a*) Dilute carbolfuchsin.
   (*b*) Methylene blue.
   (*c*) Undiluted carbolfuchsin.
   (*d*) Thionin.
   (*e*) Gram stain counterstain with dilute carbolfuchsin.

V: 1. In hanging drop are observed:

(*a*) Bacillary-forms rod-shaped microorganisms of different lengths, often in chains, often in filaments. It is essential to distinguish between true motility, where the organism changes its position in the field, and Brownian movement, which is an oscillatory movement possessed by all small bodies (whether living or not) suspended in fluid.

(*b*) Spherical bacteria, cocci of different sizes, non-motile. They are usually single, often double (diplococci), cubic (sarcina), or quadruple (tetrads), often in chains (streptococci), or in irregular clumps (staphylococci).

(*c*) Spiral or curved bacteria are either very small or extremely large, very motile, and can be observed only at the edges of the preparation, where they usually remain steady for a few seconds.

(*d*) Protozoa. Ciliata usually predominate; *Paramecium*, a holotrichous Ciliate, and *Stylomachia*, a hypotrichous Ciliate, are mostly present in hay infusions. Occasionally representatives belonging to the Sarcodina and Mastigophora can be noted. Sketch the various forms; for a careful study of the structure, arrangement of cilia, cytostome, contractile and digestive vacuoles, narcotize the animals by adding a trace of chloretone to the hanging drop. The following references give illustrations which may be useful in the identification of the various protozoa:

Stokes. *Aquatic Microscopy.* New York, J. Wiley and Sons, 1918.

Ward and Whipple. *Fresh Water Biology.* New York, J. Wiley and Sons, 1918.

(*e*) Débris. Open the diaphragm and the entire picture will disappear. Keep the slide until the end of the exercise and compare

with the stained films. Always keep in mind that unstained preparations only satisfactorily demonstrate the motility of bacteria. The preparation is destroyed in the following way: By means of forceps, the cover slip is carefully taken away from the slide and is put in the lysol solution. Avoid the possibility of the droplet touching the edges of the vaseline ring. The slide is dipped in benzol and cleaned with a towel.

PROBABLE MISTAKES:

1. When moving up and down, the cover slip may become detached from the slide. Cause: The cedar oil is too thick or the vaseline too thin.

2. When focusing, the bacteria appear indistinct. Cause: The drop is too thick.

3. The field is dark and the bacteria are indistinct. Cause: The objective is not in focus, or a wrong lens is used.

4. The bacteria are surrounded by light areas. Cause: The drop is dry; the vaseline seal is not air-tight.

5. Large, light crystal-like figures appear. Cause: The vaseline has been focused.

6. No bacteria or débris visible. Cause: Too much light; close diaphragm.

V: 2. Examination of Stained Preparations.

A minute amount of material is taken up with the platinum wire loop and placed on a slide which is held by forceps. By means of the curved needle it is spread gently over the surface in order to avoid distortion of the fine structures. Flame the loop. Let the preparation dry, which may be easily accelerated by holding the film high above the flame. When it is dry fix the bacteria by passing the slide, film upward, three times through the middle of the upper half of the gas flame. In this manner the bacteria become fixed (adherent by the coagulation of albuminoids), and if one is not dealing with spores, they are killed. When urine or other fluids, which contain no albuminoids, are examined, it is advisable to mix the material with a small amount of egg albumen. Prepare at least six different slides in the same manner. The fixed and cooled preparations are ready for staining with undiluted and diluted carbolfuchsin, methylene blue, thionin, and by Gram's method. Examine the stained films with the immersion lens, flat mirror, open diaphragm and best light.

1. Observe in the slide stained with diluted carbolfuchsin, bacilli, cocci, spirilli, and débris. The bacteria are intensely red; besides these, poorly stained organisms are also visible. Why?

2. In the preparation stained with methylene blue, the bacteria and the débris are far more distinctly visible than in the first prepara-

tion. This is characteristic of methylene blue stain and it can therefore be recommended for all specimens where coagulable material, tissue, serum, etc., surround the bacteria.

3. In the preparation stained with undiluted carbolfuchsin, the débris is stained very heavily and obscures practically all the bacteria. The specimen is overstained. Dilute solutions applied for longer periods are better stains than concentrated stock solutions. Why are concentrated stock solutions used?

4. In the preparations stained with carbol thionin, the bacteria are stained dark blue, often with a violet tinge. How is the débris stained? Is the stain polychromatic and specific for certain structures of the microorganisms?

5. In the preparation stained by Gram's method note the difference in the staining reactions. Sketch and explain the differences. What is meant by "Gram positive"? Name the organisms which are Gram negative.

VI. Qualitative Examination of Aerobic Bacteria in the Air.

4th day. The purpose of this exercise is to emphasize the possibilities of aerial contamination and the necessity for proper care in avoiding and detecting this source of error in bacteriologic technic. This experiment may be considered roughly quantitative and should bring out interesting observations concerning the temperature relations of some of these organisms. The experiment cannot be considered a success unless the control plates remain sterile.

1. *Pour melted agar* aseptically into 4 Petri dishes and allow to harden. Be sure to let the agar cool to 40°–42° C. before pouring, otherwise the covers of the Petri dish will become too moist and the surface of the agar will have to be dried as described on page 117 before the exposures are made. The plates may be poured the day before use, inverted in the locker over-night, and the moist covers exchanged for dry, sterile ones. This prevents spreading. Glazed clay covers used in the same way are even better. If incubated with clay covers, however, the media may become too dry.

2. The time of exposure may be varied. It is suggested that exposure of 5, 10, and 20 minutes be made in the laboratory by different students and the results compared. For variety's sake it would be interesting if some of the students in a group expose their plates respectively in the animal room, the media kitchen, a recently swept room, a closed cabinet, a crowded lecture room, the incubator room, or in the open air on the campus (exposed to sunlight or in the dark). Keep two plates poured at the same time but unexposed, as controls.

Second Week

VI (*Continued*). Examination of the plates exposed to air.
VII. Examination of yeasts.
VIII. Examination of molds.
IX. Study of pure cultures.
X. Isolation and cultivation of bacteria.

Special Materials Required—

Lacto-phenol solution.
Neutral red 1:1000 in salt solution.
Saturated aqueous solution of methylene blue.
Agar slants (No. 57).
Agar "deeps" (No. 57).
Gelatin (No. 22).
Bromcresol purple milk (No. 14).
Peptone broth (No. 41 or 58).
Potato blocks (No. 61).
Glucose, Lactose, Mannite, Saccharose } Peptone solution containing 1 per cent Andrade's indicator and small fermentation vials (No. 15).

Cultures—

Cakes of yeast.
Pure cultures of yeast.
Pure cultures of mold.
Molds grown on bread, leaves, fruit, dry bread.
Pure culture of *B. coli* suspended in broth.
Pure culture of *B. fluorescens* suspended in broth.
Mixed culture of *B. pyocyaneus* and *Staphylococcus albus* in broth suspension.

VI. After incubation at room temperature or 37° C. (control plates at the same temperature) count the number of colonies which have developed; note the color, form, and consistency; determine the type of organism by Gram's stain, and record the results in the following manner:

TABLE 5
Air Bacteriology

Time of exposure	Incubation	Count of colonies	Gram stain	Morphology	Bacteriological Record No.
5 minutes	Room				
	37° C.				
20 minutes	Room				
	37° C.				
Not exposed	Room				
	37° C.				

For detailed methods of bacteriological air examination consult the following references:

ROSENAU, M. J. *Preventive Medicine and Hygiene.* Ed. 4; New York and London, D. Appleton and Co., 1923, p. 938.

Committee Report in *Am. Jour. Pub. Health,* 1917, 7, p. 54.

2d day. Make duplicate transplants of 2 typical colonies on to an agar slant, a potato block and into a tube of peptone broth. Incubate one of each at room temperature, the other at 37° C. Examine the 24-hour broth cultures for motility. Is there any difference in the growth at room temperature and at 37° C? What is the value of potato medium? Continue the observations of the transplants throughout the week.

VII. Examination of Yeasts.

1st day. Pure cultures of yeast and cakes of baker's yeast will be supplied.

1. Make hanging drop preparations of the pure culture and of the baker's yeast in .85 per cent saline.

2. Vital staining: make hanging drop preparations of the pure culture and the baker's yeast in a dilute neutral red or methylene blue solution. Prepare some films and stain with carbolfuchsin, methylene blue, and by Gram's method. Note the shape, arrangement and size, mode of reproduction. Observe the nucleus, cell wall, vacuoles, granules, and spores. Are they motile? What type of nuclear division is present? Which method of staining gives the best results? What are true and false yeasts?

3. Study the intracellular spore formation of the pure culture. Proceed in the same manner as with the hanging drop preparation but allow the drop to dry before placing it over the hollow in the slide, and seal it with vaseline. Incubate at 37° C. or at room temperature for 3–4 days and examine daily. Note the appearance of spores. How many are there? Where are they formed? When spores have developed remove the cover slip, stain with warm carbolfuchsin and partially decolorize with 0.5 per cent aqueous acetic acid solution. What is the color of the spores?

Reference:

ANDERSON, H. W. Yeast-like fungi in the human intestinal tract. *Jour. Infect. Dis.,* 1917, 21, p. 341.

VIII. Examination of Molds.

1st day. Organic infusions, culture media, old milk, etc., are likely to contain the growth of molds. Select small fragments for moist preparation in water or preferably in "lacto-phenol." Place on a slide a drop of lacto-phenol; tease in the solution a fragment of the mold and protect it with a cover slip.

In case the mold growth is very dry scrape it off with a scalpel, transfer it to the slide in a drop of 95 per cent alcohol with a few drops of N/10 NaOH or ammonia. Tease the specimen, add a drop of glycerin, cover with a cover slip.

Specimens are also fixed to slides; the air-dry preparations are treated with methyl alcohol and stained with dilute methylene blue and by Gram's method. A careful study of these preparations is most important in order to recognize molds which may be encountered as accidental contaminations in the course of subsequent exercises. Pure cultures on bread, and plum infusion, are examined in the same manner. They will be used in order to acquaint the student with the principal morphological characters of taxonomic importance. Prepare sketches showing the character of the hyphae, especially whether septate or non-septate; note the location of the nuclei, conidia, etc.

Recognize the following groups:

Ascomycetes:

Aspergillus: Frequent contaminant of culture media, occurring as a felted yellow green, or block mold growth; rows of spores or 'conidia' develop from finger-like processes or 'sterigmata' borne on an expanded aerial septate hypha.

Penicillium glaucum occurs as a dull green growth on food and mediums. The one-celled conidia are arranged in chains, which are born by 'sterigmata.' The much branched 'conidiophores' are not inflated, sharply differentiated from the mycelium, and form broom-like structures.

Phycomycetes:

Mucor mucedo exemplifies the group. Mycelium made up of non-septate hyphae; asexual spores developed in a spherical apical 'sporangium' or spore case born on the expanded end of an aerial hypha; these 'sporangiophores' are not clustered nor dichotomous and are gray or brownish without metallic luster. A columella is present. Under certain conditions 'zygospores' are also formed as the result of conjugation of two hyphae at their tips.

2d day. 1. Moisten some bread with sterile water, place in a clean high beaker and leave at room temperature for 4 days. Note and study the molds which develop.

2. Specimens of molds on leaves, fruit, and cheese will be furnished. Examine them in handing drop and in stained preparation. Identify the family and if possible the species.

Reference:

Buchanan, E. D., and Buchanan, R. E. *Household Bacteriology.* New York, Macmillan, 1913, pp. 66–84.

IX. Study of Pure Cultures—*B. coli* and *B. fluorescens.*

2d day. 1. Make a Gram stain and hanging drop preparation of each culture supplied and make a transplant to an agar slant.

3d day. Make a Gram stain of the culture which has grown in the transplant made yesterday. If the culture is pure, make transplants of each culture to the following set of media:

1 peptic digest broth.

1 cresol purple milk.

2 potato slants, incubate one at room temperature, the other at 37° C.

2 tubes of Dunham's peptone broth for indol tests. Incubate both at 37° C.

2 tubes of nitrate broth for the reduction of nitrates to nitrites. Incubate at 37° C.

1 gelatin stab.

1 tube each of glucose, lactose, saccharose and mannite broth containing 1 per cent Andrade's indicator small fermentation vials.

4th day. Note the appearance of the cultures. Allow to incubate for at least a week and note the differences in growth.

X. Isolation and Cultivation of Bacteria.

1st day. A mixed culture of *B. pyocyaneus* and *Staphylococcus albus* or *aureus* will be furnished.

1. Make Gram stain.

2. Make dilution plates. (See p. 34.) Use a very small amount of the material and make 3 dilutions. Incubate at 37° C. for 24 hours.

2d day. Note the number and structure of the colonies that have formed. Make Gram stains of several isolated colonies. Are the organisms found in a single colony *all* of *one* type? If so transfer a selected colony of each type on an agar slant. Incubate at 37° C. for 48 hours. If not, continue purification according to the method outlined on page 36.

3d day. Make a Gram stain of the growth which has developed on the agar slants inoculated with *B. pyocyaneus* and *Staphylococcus albus.* If the growth is pure inoculate into the mediums listed under IX. Incubate at 37° C. for 1–5 days. Observe the development of pigment in the pyocyaneus culture. Extract the pigment by shaking with chloroform. Is the pigment soluble in ether or alcohol? What is it chemically? How is it formed? What are the optimum conditions to demonstrate the production of pigment?

References:

JORDAN, E. O. The production of fluorescent pigments by bacteria. *Bot. Gaz.*, 1899, 27, p. 19.

JORDAN, E. O. Bacillus pyocaneus and its pigments. *Jour. Exper. Med.*, 1899, 4, p. 627.

AUBEL, E., AND COLIN, H. Action des sucres sur la fonction pigmentaire du bacille pyocyanique. *Compt. Rend.*, 1913, 75, p. 25.

KLIGLER, I. J. A biochemical study of certain bacterial pigments. *Biochem. Bull.*, 1914, 3, p. 451.

TANNER, F. W. A study of green fluorescent bacteria from water. *Jour. Bacteriol.*, 1918, 3, p. 63.

4th day. Observe and describe the growth in the tubes inoculated yesterday with *B. pyocyaneus* and *Staphylococcus albus.* Tabulate the results. Reincubate the tubes except the gelatin for 2–3 days longer. Has the gelatin been liquefied? Test for the presence or absence of liquefaction by placing the tube in the ice chest for one hour. Test the gelatin for the presence of gelatinase in the following manner: To 4.0 c.c. of melted gelatin add 1.0 c.c. of a 5 per cent solution of phenol. Mix thoroughly. Then add 5 c.c. of the 1-day liquid gelatin culture and incubate at 37° C. Note the effect after 4 and 16 hours' incubation. Has any growth taken place? What causes the liquefaction of gelatin?

Third Week

X. Continuation of the study of the products of metabolism of *B. coli, B. pyocyaneus,* and *Staphylococcus albus.*

XI. Preparation of culture media.

XII. Experiments in disinfection.

Special Materials Required—

Chemicals: Solutions to determine the presence of ammonia, indol, hydrogen sulphide, nitrites.

Beef extract.

Peptone.

Agar.

Buffer solutions P_H 6.4–7.8 with brom thymol blue or phenol red indicator.

Monobasic potassium phosphate (KH_2PO_4) and dibasic sodium phosphate ($Na_2HPO_4+2H_2O$) Sorensen's phosphate.

(For special materials required for the disinfection experiments see individual exercises.)

X. Continuation of the Study of the Products of Metabolism of *B. coli, B. prodigiosus, B. pyocyaneus,* and *Staphylococcus albus.*

1st day. Test the Dunham's peptone tubes planted last week for indol, and the nitrate broth for the reduction of nitrates to nitrites, and the plain infusion broth for the formation of NH_3 and H_2S. *Use uninoculated tubes as controls.* For the composition of the reagents see Appendix A.

3d day. Repeat the indol and nitrite tests on a new set of cultures.

XI. Preparation of Culture Media.

Before beginning this exercise carefully read pages 23–31.

1st day. 1. Prepare 1000 c.c. of beef extract peptone broth, adjust reaction to P_H 7.2 according to the method outlined on page 27. Take 250 c.c. of the broth after it has been adjusted and add 5 grams of agar to it. Boil until the agar has dissolved. Make up to volume and filter through a double layer of cotton as directed.

Check the reaction. Distribute 100 c.c. of the filtered agar in 9 c.c. amounts into test tubes and the other 150 c.c. in a flask. Cap flask with paper. Why? Sterilize in the autoclave for 30 minutes at 15 pounds pressure. The agar must be placed in the autoclave while hot; otherwise 30 minutes at 15 pounds pressure will not be sufficient to insure sterilization. Why?

2. Autoclave the 750 c.c. of broth for 30 minutes at 15 pounds pressure.

3. Prepare 200 c.c. sterile buffered saline solution. See Appendix A.

4. Test the sterility by incubating the *sterilized* agar for 48 hours at 37° C.

2d day. Filter the broth through filter paper. (The process is hastened by first heating the broth.) Tube some of it (100 c.c.) in 9 c.c. (accurately measured) amounts in test tubes and about 50 c.c. in approximately 5 c.c. amounts. Measure the remainder into small flasks. Cap flasks with paper. Autoclave for 30 minutes at 15 pounds pressure and incubate for 48 hours to test the sterility. Always keep records of the final P_H value of the media. Never forget to resterilize the unused portion of a batch of medium.

These media will be used in the experiments on anthrax and later for the preparation of blood agar plates.

XII. Experiments in Disinfection.

3d week. Before attempting to carry out the experiments in disinfection read over the experiment assigned carefully, and assemble all the material necessary to execute the tests.

A. Resistance of bacteria to drying.

B. Resistance of bacteria to heat.

C. Resistance of bacteria to chemical agents.

a. Chloroform
b. Formalin
c. Liquor cresolis compositus
d. Mercury bichloride
e. Neutral acriflavine
f. Mercurochrome
g. Gentian violet.
h. Brilliant green

D. Standardization of disinfectants. Determinations of the 'phenol coefficient.'

E. Bactericidal effect of sunlight.

F. Disinfection of the hands.

G. Disinfection of excreta.

H. Disinfection of water.

A. Determination of Resistance of *Staphylococcus aureus*, *B. coli*, and *B. subtilis* against Drying

SPECIAL MATERIALS REQUIRED—

A desiccator with $CaCl_2$ or phosphorus pentoxide.
Filter paper cut in small squares (about 5 × 5 cm.) placed in Petri dishes and sterilized in the same way as the cotton or silk threads.
Sterile Petri dishes.
Sterile test tubes.
3 sterile filter tubes.
36 silk or cotton threads cut in lengths of two centimeters, placed in Petri dishes and sterilized in the autoclave at 15 pounds pressure for 30 minutes.
8–10 peptic agar "deeps" (No. 57).
Peptic digest agar slants.
Peptic digest broth (No. 58).
Sterile saline.
24-hour cultures of *Staphylococcus aureus* grown on plain agar.
24-hour cultures of *B. subtilis* grown on plain agar.
24-hour cultures of *B. coli* grown on plain agar.

1st day. 1. Prepare all of the material needed for the experiment.

2. Make transplants of the stock cultures of *Staphylococcus aureus, B. subtilis,* and *B. coli* supplied to peptic digest agar slants and incubated for 48 hours at room temperature, or at 37° C. for 18–24 hours. Why?

3. Pour about 4 agar plates and incubate for 24 hours to test the sterility. The other agar plates are poured when needed.

2d day. 1. To have the bacteria in a hardy and suitable condition for the resistance tests, they are best dried on *silk or cotton threads.* Prepare suspensions of *Staphylococcus* and *B. subtilis* and *B. coli* as follows: Carefully add 4–5 c.c. of *sterile saline* to each of the cultures. Emulsify the culture, taking particular care to avoid digging into the agar. Then transfer the *Staphylococcus* culture to *one sterile filter,* the *B. subtilis* culture to the second and the *B. coli* culture to the third, and filter into a *sterile test tube.* After filtration examine each suspension microscopically to determine whether aggregates of bacteria are present and if spores of bacteria are present in the *B. subtilis* culture. Shake the suspension thoroughly until the aggregates are broken up. Place separately in *sterile Petri dishes* from 2 to 3 c.c. of the bacterial suspension. Twelve silk threads are placed in each suspension. As soon as they are soaked with the fluid they are removed singly by means of sterile forceps and are dried on *sterile filter paper*—care being taken that separate sterile forceps are used for each culture. The threads are kept separately in *sterile Petri dishes.*

One silk thread of each suspension is placed on an agar plate and serves as a control. The other silk threads are dried in a *desiccator* and are held in the ice box.

3d day. The silk threads in the desiccator should be dry. The control plates should show abundant growth. Transfer one thread of each of the bacterial species to an *agar plate,* taking care that none of the threads touch each other. Incubate at 37° C.

4th day. Note growth of the organisms dried for one day. If growth is poor or absent, reincubate.

5th day. Place another of each of the threads on an *agar plate,* incubate and note growth after 24 to 72 hours. Repeat this test after the fifth, the sixth, the eighth, the ninth, and the twelfth day of drying. Test for complete destruction of the bacteria desiccated by transferring the last dried thread to a tube of *peptic digest broth.* Why in a liquid medium?

B. Resistance of Bacteria to Heat

MATERIALS REQUIRED—

Desiccator.
Filter paper cut in small squares (about 5 × 5 cm.) placed in Petri dishes and sterilized in the same way as the silk threads.
3 sterile beakers 200–250 c.c. capacity.
3 sterile filter tubes.
Sterile Petri dishes.
60–70 sterile silk threads cut in lengths of 2 centimeters, placed in Petri dishes and sterilized in the autoclave at 15 pounds pressure for 30 minutes.
1 flask containing about 400 c.c. sterile distilled water.
Sterile water in test tubes approximately 10 c.c. amounts.
5 tubes peptic agar "deeps" (No. 57).
Peptic digest broth in small tubes (No. 58).
24-hour agar slant culture of *Staphylococcus albus.*
24-hour agar slant culture of *B. coli.*
24-hour agar slant culture of *B. subtilis.*
Compact parcel containing coat or towels.

1st and 2d days. Proceed as under A.

3d and 4th days. (1) Resistance against Temperature of 60° C.

Pour a layer of *melted agar* into five Petri dishes. Place three test tubes containing *10 c.c. sterile water* in a water bath which is kept constant at a temperature of 60° C. A fourth tube holds a thermometer. Six silk threads of each of the bacterial types are placed into each of the tubes respectively. As controls transfer one thread each on an agar plate. At intervals of 2, 10, 15, 30, and 60 minutes, and of 2 hours, two of the silk threads each are

removed by means of a long curved sterile bent glass capillary tube (made from a Pasteur pipette). One is placed on a sector of the agar plate, the other in a small *tube of sterile broth.* Flame the capillary tubing carefully between each manipulation. Incubate and examine daily. Tabulate and interpret results.

(2) Resistance against Boiling

Three small beakers each containing about 100 c.c. of water are brought to the boiling point; keep the water at the same level by adding hot water when necessary. Immerse 12 silk threads of *Staphylococcus,* 12 of *B. coli,* and 14 of *B. subtilis* in the three beakers respectively and at intervals of 1, 2, 5, 7, 10, and 15 minutes in the case of *Staphylococcus aureus* and *B. coli,* and 30, 60, 120, 180, 240, 300, 360, 420, and 480 minutes in the case of the *B. subtilis,* 2 of each of the threads are removed. One is placed on the surface of a properly labelled agar plate and one in a tube of broth. Incubate all plates and broth and examine daily for the next 4–5 days. Tabulate and interpret the results in the day book.

(3) Sterilization of Clothing, etc., in Flowing Steam

1st and 2d days. Proceed as outlined under "A."

3d and 4th days. Large articles, such as clothing, linen, etc., cannot be boiled and must therefore be *sterilized by steam.* For this exercise the infected silk threads, wrapped in filter paper together with a maximum thermometer, are placed in the middle of a small parcel containing the coat or towel which is to be sterilized. The entire parcel is placed in the steam sterilizer. A few silk threads and a piece of paper written upon with ink are placed in an old book (this book will be destroyed), also an old shoe or a piece of leather is placed in the sterilizer together with the book. A thermometer inverted in the steam sterilizer will indicate the temperature surrounding the articles that are being sterilized. To determine the temperature inside these parcels, it is advisable to have a maximum contact thermometer which will indicate on starch-iodine control paper when the temperature of 98° C. has been reached. For details on heat penetration consult Bulletin No. 16L, National Canners' Association. As soon as the sterilizer has reached a temperature of 100° C. (note the so-called coming up time) the exhaust valve is closed and the autoclave kept at 100° C. for 20 minutes. The steam is then turned off and the articles taken out for inspection and bacteriologic examination.

The book is spoiled, the cover demolished, but the ink has not disappeared from the written paper. The leather is hard and useless, the clothing has not suffered. The silk threads are cultured on agar plates.

Another series of silk threads is placed in clothing or, better, in cotton parcels exposed to a temperature of 100° C. in a hot-air sterilizer. At intervals of 2, 5, 10, 30, and 60 minutes, one of the silk threads each is taken out and placed on an agar plate. Incubate at 37° C. and record results after 24 hours' incubation and again at frequent intervals for the next 7 days.

C. Resistance of Bacteria to Chemical Agents

(*a*) Chloroform.
(*b*) Formalin.

MATERIALS REQUIRED—

0.1 N ammonium hydroxide.
Chloroform.
Formalin.
Desiccator.
Sterile filter paper cut in small squares (about 5 × 5 cm.) placed in Petri dishes and sterilized in the same way as the silk threads.
3 sterile filter tubes.
Sterile Petri dishes.
10–12 silk threads cut in 2 cm. lengths, placed in Petri dishes and sterilized in the autoclave at 15 pounds pressure for 30 minutes.
Sterile distilled water—approximately 200 c.c.
Peptic digest agar "deeps" (No. 57).
Peptic digest agar slants.
Peptic digest broth (No. 58).
24-hour agar slant culture of *Staphylococcus aureus.*
24-hour agar slant culture of *B. coli.*
24-hour agar slant culture of *B. subtilis.*

1st day. 1. Prepare and sterilize the silk threads, filter paper, filter tubes as described under "A."

2. Make a transplant of the cultures supplied of *Staphylococcus aureus, B. coli,* and *B. subtilis.*

3. In addition to the cultures planted on agar slants, make surface streak plates of each culture. Proceed in the following manner: Pour melted, cooled (45° C.) agar into three Petri dishes. Allow to harden. Place a small drop of the *B. coli* culture supplied on the surface of the agar on one of the plates. By means of a 'dalli' spread the culture over the surface of the plate. Similarly make surface streak plates of *Staphylococcus aureus* and *B. subtilis.* Incubate 24 hours.

2d day. 1. Soak silk threads in bacterial emulsions as described under "A."

2. Determine the resistance of bacteria to (a) *chloroform.* The agar plates prepared yesterday are inverted and at the bottom of each Petri dish is placed a piece of cotton soaked with chloroform. Thirty minutes and again an hour after the exposure to the chemical a subculture in glucose broth is made. Incubate the broth at 37° C. and note growth daily or every 48 hours for the next 7 days.

3d day. Determine the resistance of bacteria to (b) *formalin.*

If there is a room at disposal for this exercise, formaldehyde is generated in it in the following manner:

Mix for each cubic foot of air space, 0.5 c.c. of formalin with 0.25 gram of potassium permanganate. The permanganate is first placed in a tin can and the formalin poured into it.

Close all the openings of the room (ventilators, windows, keyholes, etc.) with cotton soaked in *mercury bichloride.* In a few drawers of the room, or in suspended clothing or laboratory coats, silk threads prepared with *Staphylococcus* and with *B. subtilis* are placed. If possible, place also for this exercise some silk threads in a *closed box* or in a drawer which will be kept closed. After 4 to 10 hours open the windows and, when the formalin has evaporated, enter the room, collect the threads, wash them in a weak solution of *ammonia,* then in sterile water, and place them in broth and on agar plates.

C(*c*). Determination of Resistance of *Staphylococcus aureus* and *B. subtilis* to 5 per cent *liquor cresolis compositus*

MATERIALS REQUIRED—

10 per cent solution of liquor cresolis compositus.
Sterile Petri dishes.
Small flask of sterile water.
Peptic digest agar "deeps" (No. 57).
Peptic digest broth (No. 58).
1 24-hour agar slant culture of *Staphylococcus aureus.*
1 48-hour agar slant culture of *B. subtilis.*

1st day. Make transplants to peptic agar slants of the *Staphylococus aureus* and *B. subtilis* culture supplied. Prepare the 10 per cent *liquor cresolis compositus* solution, distilled water, etc.

2d day. 1. Examine the *Staphylococcus* and the *B. subtilis* culture for purity. Make another transplant of the *subtilis* culture and incubate for 48 hours.

2. Determine the resistance of *Staphylococcus aureus* in 5 per cent liquor cresolis compositus in the following manner:

Twelve agar tubes are liquefied and kept at a temperature of 45°–50° C. At the same time twelve broth tubes are kept ready. A few loopfuls of the *Staphylococcus* culture are carefully emulsified in a test tube containing 2 c.c. of sterile water until it is definitely turbid. Clumps must be avoided. Add to the suspension 2 c.c. of the 10 per cent disinfecting solution—this gives a final dilution of 5 per cent. Immediately after mixing, one loopful of this mixture is transferred to a tube of melted agar and one loopful is planted in a tube of broth. The agar is thoroughly mixed and poured into a sterile Petri dish. The same process is repeated at intervals of 2, 5, 10, 15, and 30 minutes. Prepare duplicates, i.e., pour 2 plates and inoculate 2 broth tubes at each period. After the agar in the plates has hardened, invert the Petri dishes and incubate them and the broth at 37° C. for 48 hours.

3d day. 1. Determine the resistance of *B. subtilis* in a 5 per cent "liquor cresolis compositus" solution. Melt 20 tubes of agar and have ready 20 tubes of broth. Follow the same procedure as given for the *Staphylococcus* culture but remove samples after 5, 15, 30, 40, 50, 60, 80, 120, and 180 minutes.

2. Examine the broth cultures and count the number of *Staphylococcus* colonies that have developed on the agar plates incubated at the last period. Is there any growth in the broth when the agar plates are apparently sterile? If so, why? It should be noted that the broth does not permit a quantitative determination of the destructive action of the "liquor cresolis." In all instances in which unexpected growth has developed, determine whether or not this abnormal growth is caused by germs in the atmosphere, a point which can usually be decided without any difficulty by inspection of the agar plates. Place the cultures back in the incubator and reëxamine the next day and again after 3–4 days' further incubation. Explain results.

C(*d*). Deterination of the Resistance of *Staphylococcus* and *B. subtilis* to *Mercury Bichloride Solution*

MATERIALS REQUIRED—

0.1 N ammonium sulfide solution.

10 c.c.—1 per cent mercury bichloride solution.

Desiccator.

Filter paper cut in small squares (about 5 × 5 cm.) placed in Petri dishes and sterilized in the same way as the silk threads.

Sterile filter tubes.
Sterile Petri dishes.
24 silk threads cut in 2 cm. lengths, placed in Petri dishes and sterilized in the autoclave at 15 pounds pressure for 30 minutes.
1 flask (200 c.c.) sterile distilled water.
4–9 c.c. sterile distilled water blanks.
16–20 9 c.c. plain peptic broth.
24 tubes plain peptic broth (3–4 c.c. amounts) (No. 58).
Peptic digest agar "deeps" (No. 57).
Peptic digest agar slants.
24-hour agar slant culture of *Staphylococcus.*
24-hour agar slant culture of *B. subtilis.*

1st day. Prepare the silk threads, filter tubes, filter paper, 9 c.c. broth blanks, saline blanks, etc. Make transplants to agar slants of the *Staphylococcus aureus* and the *B. subtilis* culture.

2d day. 1. Make another transplant of *B. subtilis* to an *agar slant.*

2. To test the efficacy of *mercury bichloride* make the following dilutions:

Dilute the 1 per cent mercury bichloride 10 times, 100 times and 1,000 times. The following dilutions are then obtained:

1:100 (original solution).
1:1,000.
1:10,000.
1:100,000.

Add 1 c.c. of the above dilutions to each 9 c.c. *broth blank.* Make four sets of each dilution. Emulsify in 2 tubes of each dilution a large loopful of the *Staphylococcus* and in the other 2 loopfuls of the *B. subtilis* culture; mix thoroughly. Place one of each set in the incubator, keep the other at room temperature. Observe daily and record the results.

3d day. 1. Examine the $HgCl_2$ broth cultures. In the broth tube in which the concentration of $HgCl_2$ is the highest do not mistake albumen precipitates for growth. Is the disinfectant action more pronounced at 37° C. or room temperature? What effect has an increase in temperature on the action of the disinfectant? Determine in the tubes which show no growth whether the apparent sterility is caused by antisepsis, i.e., mere inhibition of the development of the bacilli, or whether the organisms have actually been destroyed.

2. Prepare silk threads as described under "A," using the *B. subtilis* culture planted 2 days ago. Examine the suspension for the presence of spores.

4th day. The silk threads placed in the *desiccator* should be dry. Determine the bactericidal effect of small amounts of $HgCl_2$ which might be carried over to the culture media by carrying out the following experiment:

1. Melt 4 tubes of agar, and after cooling pour plates.

2. Place 24 dried silk threads, previously soaked in the *B. subtilis* suspension, in a 1:1,000 mercury bichloride solution. After 2, 5, 10, 15, 30, and 60 minutes respectively remove four silk threads. Two of these are carefully washed in sterile water. After washing one is placed on the surface of the agar plate, the other in the peptic digest broth. The two other threads are washed in dilute ammonium sulfide solution. What is the reaction that takes place? Place one of these threads on the surface of an agar plate, the other in a tube of peptic digest broth. Incubate at 37° C. Examine after 24 hours and again after three days' incubation. Explain the results.

C(*e*). Antiseptic Potency of Acriflavine in Peptone Water

MATERIALS REQUIRED—

100 c.c. volumetric flask.
Acriflavine.
Approximately 32 sterile test tubes (small size), 100 × 12 mm.
Sterile distilled water.
Sterile peptone water about 150 c.c.
Peptic digest agar slants (No. 57).

1st day. 1. Plant one tube each of peptone water with

(*a*) *B. coli.*

(*b*) *Staphylococcus aureus.*

2. Fill a series of small test tubes aseptically with 4.5 c.c. of sterile peptone water. About 30 tubes will be needed for the test. Steam the tubes in the Arnold sterilizer for 20 minutes. When cool place them in the incubator to test the sterility.

2d day. 1. Make up the following dilutions of acriflavine in sterile distilled water:

1:500	1:20,000
1:2,000	1:40,000
1:10,000	1:100,000
1:20,000	

Weigh out carefully on a chemical balance 1 gram of acriflavine; place it in a volumetric flask and dissolve it in 75 c.c. of distilled water (P_H 7.0); when dissolved make up volume.

From this stock solution the various dilutions are prepared as follows:

1. 1:500 = 0.5 c.c. of 1:100 stock solution + 2 c.c. of distilled water.
2. 1:1,000 = 1.0 c.c. of 1:100 stock solution + 9 c.c. of distilled water.
3. 1:2,000 = 1.0 c.c. of 1:1,000 stock solution + 1 c.c. of distilled water.
4. 1:10,000 = 1.0 c.c. of 1:1,000 stock solution + 9 c.c. of distilled water.
5. 1:20,000 = 1.0 c.c. of 1:10,000 stock solution + 1 c.c. of distilled water.
6. 1:40,000 = 1.0 c.c. of 1:10,000 stock solution + 3 c.c. of distilled water.
7. 1:100,000 = 1.0 c.c. of 1:10,000 stock solution + 9 c.c. of distilled water.

2. Inoculate 14 tubes of peptone water measured in 4.5 c.c. amounts with a loopful of the 24-hour peptone water culture of *Staphylococcus aureus*. Likewise inoculate another set of 14 tubes of peptone water with a loopful of a 24-hour peptone water culture of *B. coli*.

3. To each of 6 tubes from each set add 0.5 c.c. of the various dilutions of acriflavine respectively. The seventh tube serves as a control. Incubate the complete series of tubes at 37° C. for 48 hours.

3d day. 1. Note growth and determine the efficacy of the acriflavine by testing the sterility or the degree of inhibition of the cultures grown in each dilution of the dye by making transplants from each dilution to a peptic digest agar slant. Use two loopfuls for each transplant. Incubate the slant for at least 48 hours.

2. To the 2 sets of tubes inoculated the preceding period with *Staphylococcus* and *B. coli* respectively but to which no acriflavine has been added, add 0.5 c.c. of the series of dilutions of acriflavine making up fresh dilutions. Incubate for 24 hours.

4th day. 1. Test the efficacy of the dye in sterilizing the *B. coli* and *Staphylococcus* cultures by making a transplant from each tube to a peptic digest agar slant. Incubate at 37° C. for 2–3 days. Note growth. The result of this experiment should be compared with that obtained in the next series.

C(*e*). Antiseptic Potency of Acriflavine in Sterile Serum or Sterile Urine

Proceed in the same manner as outlined for the foregoing experiment, substituting sterile horse serum or urine in place of the peptone water. Record and compare the results obtained in this with those of the preceding experiment.

References:

Browning, C. H. *Applied Bacteriology.* Oxford Press, 1918.

Browning, C. H., and Gulbransen, R. Antiseptic potency of acriflavine. *Brit. Jour. Exper. Path.*, 1921, 2, p. 95.

C(*f*). Antiseptic and Bactericidal Potency of Mercurochrome 220

Test the antiseptic and bactericidal property of mercurochrome 220 on *Staphylococcus aureus* and *B. coli* grown in peptone water and suspended in sterile distilled water. The following *final dilution* of the dye should be tested:

1:100	1:10,000
1:1,000	1:20,000
1:5,000	1:100,000

Use the same technic as described under (*d*). Make the distilled water suspension by emulsifying a small amount of a 24-hour agar slant culture of the organism in distilled water. Then add 1 loopful of this suspension to the tubes containing the requisite amounts of mercurochrome.

Reference:

YOUNG, H. H., WHITE, E. C., AND SCHWARTZ, E. O. A New Germicide for Use in the Genito-Urinary Tract, Mercurochrome 220. *Jour. Am. Med. Assoc.*, 1919, 73, p. 1483.

C(*g*). Bactericidal Action of Gentian Violet (Coleman and Bell)

1. Determine the bactericidal effect of an aqueous solution of gentian violet on *B. coli* and *B. subtilis* in the following dilutions:

1:1,000	1:100,000
1:10,000	1:300,000
1:50,000	1:200,000

Test the dye in peptone water medium following the procedure outlined under (*d*).

2. Demonstrate the bactericidal action of gentian violet by the divided plate method of Churchman.

SPECIAL MATERIALS REQUIRED—

3 sterile Petri dishes provided with a metal strip about 0.5 cm. wide and long enough to divide the Petri dish in two compartments.
Peptic agar measured in 9 c.c. amounts (No. 57).
Culture and dye dilutions.

2d day. Make dilutions of 1:10,000; 1:50,000; 1:100,000 respectively of gentian violet in melted, cooled (45° C.) agar; keep in water until ready for use. After the Petri dishes are sterilized adjust the metal strip with sterile forceps so that the dish is divided into 2 compartments. Force the strip down so that one edge touches the bottom of the dish as uniformly as is possible. Keep the dish covered as much as possible. Pour plain agar in one compartment. Allow to harden. Then pour gentian violet agar in the other compartment. The depth of the agar in the two compartments should

be as near alike as possible. After the agar has hardened remove the strip by means of sterile forceps. Carefully streak one side of the plain agar with a loopful of broth culture of *B. coli*, being careful not to dig into the agar. Then in the same relative position make a streak on the gentian violet agar with a loopful of *B. coli*. On another section of the plate inoculate first the plain agar with a loopful of *B. subtilis*, and then the gentian violet side with a loopful of *B. subtilis*. Incubate at 37° C.

3d and 4th days. Examine plates and record and explain the results.

Reference:

CHURCHMAN, J. W. The selective bactericidal action of gentian violet. *Jour. Exper. Med.*, 1912, 16 p. 221; also 1921, 33, p. 569; *Jour. Am. Med. Assoc.*, 1922, 79, p. 1657; *Jour. Urology*, 1924, 11, p. 1.

C(*h*). Bactericidal Action of Brilliant Green

SPECIAL MATERIALS REQUIRED—

Brilliant green (Grubler).
Sterile Petri dishes.
Sterile distilled water.
1 per cent glucose digest broth measured in 9 c.c. amounts.
Peptic digest agar "deeps" (No. 57).
24-hour peptic broth culture of *Staphylococcus aureus*.
24-hour peptic broth culture of *B. coli*.

2d day. Make transplants of *Staphylococcus aureus* and *B. coli* to peptic digest broth. Incubate 48 hours.

3d day. Make the following dilutions of brilliant green:

1:1,000	1:30,000
1:10,000	1:40,000
1:20,000	1:50,000

Incubate a series of 7 tubes of glucose peptic digest (9 c.c. in each tube) with one loopful of *Staphylococcus aureus*. Mix thoroughly, then inoculate a tube of melted agar with one loopful of an inoculated broth tube. Rotate the agar thoroughly and pour it into a Petri dish. This plate culture will give an approximation of the number of organisms inoculated into each tube. Likewise inoculate another series of 7 tubes of glucose peptic digest broth with *B. coli* and prepare a control plate. Then add 1 c.c. of the dilution of brilliant green to each tube. Note the final dilutions. The seventh tube in each set serves as a control. Incubate 18 hours at 37° C.

4th day. Examine the culture and prove the absence of growth in the incubated cultures.

References:

BROWNING, C. H., GULBRANSEN, R., AND THORNTON, L. H. D. The antiseptic properties of acriflavine, proflavine and brilliant green. *Brit. Med. Jour.*, 1917, p. 70.

BROWNING, C. H., AND BONNEY, V. Sterilization of the skin and other surfaces by a mixture of crystal violet and brilliant green. *Brit. Med. Jour.*, 1918, p. 562.

BERWICK, C. C. The disinfection of the oral mucosa with crystal violet and brilliant green. *Jour. of Dental Research*, 1920, 2, p. 21.

KRUMWIEDE, C., AND PRATT, J. S. Further observations on the growth of bacteria on media containing various anilin dyes, with special reference to an enrichment method for typhoid and paratyphoid bacilli. *Jour. Exper. Med.*, 1914, 19, p. 501.

D. Standardization of Disinfectants: Determination of Phenol Coefficient*

(This experiment is only for advanced students who are immunized against *B. typhosus.*)

The object of the experiment is to compare the germicidal power of an unknown disinfectant against that of a 1 per cent solution of phenol.

SPECIAL MATERIALS REQUIRED—

5 per cent aqueous solution of phenol (titrated with bromine).
A solution of a disinfectant, the germicidal properties of which are unknown.
Sterile distilled water.
5 c.c. pipettes.
1 c.c. pipette divided in tenths provided with a safety cap.
2 platinum loops. (23 U. S. standard gauge wire and a 4 millimeter loop made over a No. 14 wire U. S. gauge.)
2 platinum loops.
Standard beef extract broth (No. 42).
24-hour culture of *B. typhosus* grown on standard beef extract broth.
Incubator box to keep test solutions at 20° C.

3d day. 1. Make transplants of the cultures of *B. typhosus* to standard broth. The culture has been carried over every 24 hours for 6 successive days in the same medium.

4th day. 1. Filter the culture through sterile filter paper.

2. Make up, according to directions, 2 dilutions of phenol, and 3 of the unknown disinfectant, from the following table:

From a 5 per cent solution of phenol
10 c.c. plus 35 c.c. water = 1:90
10 c.c. plus 40 c.c. water = 1:100

From a 1 per cent solution of the unknown disinfectant
10 c.c. plus 12½ c.c. water = 1:225
10 c.c. plus 15 c.c. water = 1:250
10 c.c. plus 17½ c.c. water = 1:275

* Hygienic Laboratory Bulletin No. 82. Public Health Reports, April 12, 1918; October 17, 1919.

3. Bring the water bath to 20° C. The thermometer should be in a test tube, partly filled with water, in the bath. Place the suspension of *B. typhosus* in the bath to reach the temperature of 20° C. Place the 5 large sterile test tubes in their places in the water bath.

4. Transfer 5 c.c. of the phenol dilution (1–90) to the first large test tube on the left of the water bath. Rinse the pipette in the next dilution (1–100) and transfer 5 c.c. of it to the next tube in the water bath. With another pipette, transfer 5 c.c. of the dilutions of the unknown disinfectant to the remaining three large test tubes, working from *left* to *right*.

5. Flame both platinum loops. Add 0.1 c.c. of the culture to each of the 5 large tubes at exactly 30-second intervals, beginning at the left, and taking care that the culture does not get on the walls of the tube. Mix contents of tubes after adding cultures.

6. Thirty seconds after inoculating the last large tube, start to transfer a loopful from each large tube in the water bath to standard broth, and, for comparison, peptic digest agar slants standing in the rack, and continue without interruption at intervals of 30 seconds until all the transfers have been made. The platinum loops are flamed and used alternately. Label the first tube of each row with the disinfectant and dilution. Incubate for 48 hours and tabulate results.

EXAMPLE

Time in minutes	2½	5	7½	10	12½	15
Phenol 1–90	+	−	−	−	−	−
Phenol 1–100	+	+	−	−	−	−
Unknown 1–225	+	−	−	−	−	−
Unknown 1–250	+	+	+	−	−	−
Unknown 1–275	+	+	−	+	−	−

The carbolic coefficient is therefore $\frac{225}{90} = 2.50$.

Record results and state the limitations of the test as applied to the value of a germicide in practical application.

E. Bactericidal Effect of Sunlight

SPECIAL MATERIALS REQUIRED—

12 Petri dishes.

14 thin-walled test tubes.

24-hour culture of *Staphylococcus aureus, B. coli,* and *B. subtilis.*

1st day. 1. Thin-walled* test tubes to which about 7 c.c. of peptic digest agar have been added will be supplied. Melt the agar and

* It must be remembered that the bactericidal properties of light are greatly diminished by passage through ordinary glass. The experiment should be carried out in quartz tubes, but owing to the expense of quartz glass, ordinary thin glass has been substituted.

then prepare slants extending from the bottom of the tube to within about one inch of the cotton plug. Incubate for 24 hours at 37° C. to dry any water of condensation.

2. Make transplants of the *Staphylococcus*, *B. coli*, and *B. subtilis* cultures respectively to 3 peptic digest agar slants. Incubate 24 hours at 37° C.

3d day. With a loop inoculate the surface of four slants each with *Staphylococcus aureus*, *B. coli*, and *B. subtilis*. Protect the lower half of the test tube with black paper. Then expose the tubes to the action of the sun's rays at an open window. Expose one from each set for 15, 30, 60, and 120 minutes respectively. Incubate at 37° C. and examine after 24 hours and again after 48 hours incubation.

F. Disinfection of the Hands

SPECIAL MATERIALS REQUIRED—

Solutions of:

0.1 N ammonium sulfide
1:1000 bichloride of mercury
1:40 or 1:60 carbolic acid.
1 per cent liquor cresolis compositus.

Sterile tap water.

2 dozen wooden applicators in Petri dishes and sterilized in the autoclave at 15 lbs. pressure for 30 minutes.

Reference:

KELLY, H. H. Hand Disinfection. *Am. Jour. Obst.*, 1891, 24, pp. 1414–1419.

1st and 2d days. Sterilize wooden applicators.

3d day. Disinfection of the hands. One hand is slightly moistened with sterile water and then one-half of it is scraped with a few of the sterilized applicators, or a sterile dull steel blade. These are handled by means of sterile forceps. The applicators or scrapings are then smeared over the surface of agar plates and finally placed on them. Disinfect the hands according to one of the usual ways;* rinse off the disinfectant with sterile water; then scrape the other half of the hand with fresh applicators. If bichloride is employed, ammonium sulphide is used to neutralize the antiseptic effect of

* Though the thorough sterilization of the hands is unattainable, the greatest care and sufficient time must be devoted to rendering them as clean as possible. The nails must be cut short, all frayed skin and hang-nails removed, the grooves round the bed of the nail thoroughly freed from dirt with a nail-brush, and the hands and arms deliberately scrubbed with soap, first in lathery water, and then in running water, for 10 minutes at least. Up to this point all surgeons agree. After this, however, a great variety of practice exists. Some immerse their hands and arms in various solutions, such as carbolic acid (1:40 or 1:60); bichloride of mercury (1:1000 to 2000); biniodide of mercury (1:2000) in water or alcoholic solution; liquor cresolis composition (1 per cent). It is also customary to thoroughly rub the hands and arms with swabs soaked in methylated spirits, and follow with careful drying.

the sublimate, followed by sterile water, before specimens are taken and placed on the agar. The plates are incubated at 37° C. The plates which were smeared with material collected before the disinfection, present as a rule numerous colonies (hands, which are disinfected daily, are practically sterile). The plates charged with skin scrapings after disinfection show few or no colonies. In the latter instance determine whether these colonies originate from the applicators or whether they are atmospheric contaminations. Colonies of special type are examined microscopically and identified by special cultures.

G. Disinfection of Excreta. H. Disinfection of Water

SPECIAL MATERIALS REQUIRED—

Chlorinated lime solution "American Standard."
24-hour broth culture of *B. coli.*
Fresh stool specimen.
Peptic digest broth (No. 58).

G. Disinfection of Excreta

Mix in a beaker approximately 50 grams of feces with 100–200 c.c. of chlorinated lime solution "American Standard" containing 6 ounces of the powder to the gallon; cover with a glass plate or Petri dish. Remove at regular intervals one loopful and transfer to agar slants or broth tubes. Determine the time necessary to sterilize the feces at room temperature.

H. Disinfection of Water

Infect one liter of tap water in a large flask with 2 c.c. of a young broth culture of *B. coli;* mix thoroughly; add enough of a 0.1 per cent solution of chlorinated lime (approximately 30 per cent available chlorin per liter) to make 1 part of chlorinated lime to 200,000 parts of water. Allow to stand for 5 minutes and then remove at intervals of 10 minutes for 100 minutes 1 c.c. of the water and test by cultures in lactose neutral red broth (see Appendix A) for the presence of *B. coli.*

References:

BROWNING, C. H. *Applied Bacteriology.* London, Oxford Press, 1918.

COHEN, B. The effects of temperature and hydrogen-ion concentration upon viability of Bact. coli and Bact. typhosus in water. *Jour. Bacteriol.,* 1922, 7, p. 183.

DAKIN, H. D., AND DUNHAM, E. K. *Hand-book of Antiseptics.* New York, Macmillan and Co., 1917.

HAILER, E. Die chemischen Grundlagen der Desinfektions Wirkung. *Centralbl. f. Bakteriol.,* I. Abt., 1922, 89, Beiheft, pp. 2–15; Weyl's *Handbuch d. Hygiene.* Ed. 2; Joh. A. Barth, Leipzig, 1922, 8, pp. 861–1389.

REICHENBACH. Die theoretischen Grundlagen der Desinfektion. *Centralbl. f. Bakteriol.,* I. Abt., 1922, 89, Beiheft, pp. 15–28.

Fourth Week

XII. Complete the work on disinfection.

XIII. Study of an anthrax infection.

XIV. A. Study of a pure culture of *B. subtilis.*
B. Study of aerobic spore-bearing bacilli related to *B. anthracis.*

Special Materials Required—

Animal boards.
Glass jar with concentrated crude sulfuric acid.
Sterile Petri dishes.
Blood agar plates (No. 6).
Gelatin (No. 22).
Loeffler's serum (No. 48).
Peptic digest agar "deeps" (No. 57).
Peptic digest agar slants (No. 57).
Peptic digest broth (No. 58).
Dunham's peptone solution (No. 19).
Potato slabs (No. 61).
Dextrose, lactose, saccharose, peptone solution, containing 1 per cent Andrade's indicator, and small fermentation vials (No. 15).
Mice and guinea pigs infected with *B. anthracis.*
Culture of *B. subtilis* suspended in broth.
A boiled hay suspension incubated for 3 days at 22° C.
10 per cent formalin in physiological salt solution in specimen bottles.
Solutions for Hiss capsule stain.
Solutions for Burke's spore stain.
Solutions for Moeller's chromic acid stain.

XIII. Study of an Anthrax Infection.

Mice or guinea pigs are inoculated at the root of the tail or on the abdomen with one to one-half loopful of a culture of *B. anthracis.* (This step will have been carried out by an assistant the day before the exercise is given so that the laboratory work may be started immediately.)

1st day. Observe animal, note symptoms and local reactions. Read outline on autopsy, page 68. If the animal is dead sterilize the instruments. While they are boiling prepare the animal for autopsy in the following manner: Remove the animal with forceps from the glass jar and dip it into 5 per cent lysol solution. Pin the animal on the autopsy board. Make an incision from the

larynx to the pelvis. Dissect the skin from the abdominal muscle taking especial care not to pierce the abdominal cavity. The ribs are cut through on the right and on the left side and the anterior of the thorax is removed. To collect heart blood under sterile conditions, flame an old knife until red hot, sear with it the surface of the pericardium over the ventricle, and then collect the heart blood with the aid of a Pasteur pipette. A drop of the aspirated blood is inoculated into a tube of melted and cooled (42° C.) agar, which is thoroughly mixed and poured into a sterile Petri dish, another drop is enriched in plain broth (either infusion or peptic digest broth). At least 10 slide preparations are made from the remainder of the blood, the films are fixed and stained by Gram's method, thionin, or methylene blue, and by Hiss or Muir's capsule stain. Make enough preparations that the technic of the capsule staining may be repeated several times if necessary.

By means of fresh sterile forceps and scissors (always use fresh instruments for each organ examined) a small bit of lung tissue is removed, and a fresh agar plate is inoculated by bringing the cut surface of the tissue in contact with the agar. The material which adheres to the medium is then carefully spread over the agar plate with a dalli. If 2 agar plates are available a dilution plate should be made from the first one to insure the complete separation of the colonies. The organs of the thoracic cavity are then removed and placed in a sterile Petri dish.

With a fresh knife an incision is made into the muscle of the abdomen from the xiphoid cartilage to the pelvis. With the exception of a large spleen and a general hyperaemia, nothing particular is found. (White mice may have a large spleen under *normal* conditions. Foci in the liver may be due to *B. aertryke* infections or parasites).

Cultures of the spleen, liver, and kidneys are taken in the same manner as the lung cultures, the organs are then transferred to sterile Petri dishes, smears are made for microscopic examination and handled in the same manner as the blood smears. A group of students will use blood agar plates for the streaking of the organs. Why? Bits of the organ may also be mulsified in sterile salt solution and examined in hanging drops. Other tissues as testes, mesenteric lymph-nodes, and adrenals may also be examined in the same manner. After the organs have been removed the *carcass is destroyed.* (Mice are put in a vessel with crude sulfuric acid. Guinea pigs are carefully wrapped and placed directly in the incinerator.) The instruments are boiled preferably in an alkaline soda solution (about 1 per cent), the knives and scissors

are placed in concentrated solution of liquor cresolis compositus and the animal board is sterilized in the autoclave.

Incubate the agar plates and the broth cultures at 37° C. for 24 hours. Slices of each organ are fixed in 10 per cent formalin salt solution and kept for histological examination. The remainder is autoclaved or placed in concentrated H_2SO_4.

How does the anthrax bacillus react to the Gram stain? Are any chains present? Are capsules present? What is the function of the capsule? What is the significance of the granular structure of the organism? Save some of the stained preparations and compare the appearance of the rods found in tissues and those grown on the agar. Is the organism motile? Discuss in the daybook the autopsy findings. What is the significance of the general hyperaemia?

2d day. Examine the agar plates and broth cultures inoculated yesterday. If the agar plates have been properly made well-isolated colonies should be developed on the second dilution plate. Are the colonies of uniform type (size, form, color)? Note the colonies that have developed in the depths of the 'pour plates.' Are they of the same form as those growing in the surface of the plate? Examine the colonies under the low power of the microscope. Examine in hanging drop preparation. Is the organism motile? Can you see any spores? Stain several colonies by Gram's method and after having distinguished the anthrax colonies from those resulting from contaminating organisms stain the pathogen by means of Hiss or Muir's method. Are the organisms capsulated? Make an impression preparation of one of the characteristic surface colonies.

TECHNIC OF IMPRESSION PREPARATIONS

Clean carefully a small cover slip; with the aid of forceps place it on a well-isolated surface colony; gently press it on the agar surface and lift it carefully with the forceps. The entire colony or at least a portion of it will adhere to the cover slip. Fix the film with ether-alcohol and stain with dilute methylene blue or by Gram's method.

Note the appearance of the growth in the broth. Make a Gram stain—the culture may not be pure. What other types (morphologically) are present? What is the value of this method of isolation? If spores are present stain by Burke's or Moeller's spore stain. Compare the appearance of the organism when found in tissues and in colonies under artificial cultivation. If a colony is found which consists entirely of anthrax bacilli, i.e., if the colony *appears* to be pure

inoculate an agar slant with a minute amount of the colony. If you are not sure of the purity of the growth and if many contaminants are present on the plate purify one of the colonies by the method described on page 36. One of the purified colonies can then be used to inoculate the differential media. If spores have formed the mixture can be readily freed of non-sporulating aerobes by making an emulsion of the colony in saline and heating it at 10° C. for 15 minutes. Then streak surface plates with a loopful of the heated suspension. Incubate for 24–48 hours at 37° C. Make a Gram stain and if the colony is pure test on the differential media.

3d day. Examine the agar slant planted the preceding period for purity. If pure inoculate the following mediums with some of the growth. (Only a minute amount of the growth is necessary for each inoculation.)

Plain agar slant.
Plain agar stab.
Plain infusion broth.
Dunham's peptone solution.
Dextrose, Lactose, Saccharose } Peptone solution containing 1 per cent Andrade's indicator and fermentation vials.
Gelatin.
Loeffler's serum.
Potato slab.

4th day. Note the appearance of the growth in the mediums inoculated yesterday. Is the gelatine liquefied? Is the Loeffler's serum liquefied? Note the growth along the line of puncture in the agar stab. Note the greyish felt-like growth on the surface of the agar slant. Is there a pellicle on the broth culture. Note the consistency of the deposit at the bottom of the broth culture.

XIV. A. Study of a Pure Culture of *B. subtilis.*

2d day. A culture of *B. subtilis* will be supplied. Make a transplant to an agar slant and incubate at 37° C.

3d day. Make a Gram stain and after purity of the culture is verified inoculate the same types of media as are used for *B. anthracis.* Incubate at 37° C.

4th day. Examine cultures. Note reactions and compare the morphological and cultural reactions to *B. anthracis.* Reincubate.

XIV. B. A Study of Aerobic Spore-bearing Bacilli Related to *B. anthracis.*

A group of advanced students will search specimens of hay and soil for spore-bearing aerobes morphologically related to *B. anthracis.*

1st day. Place some hay dust or soil in a thin-walled beaker, cover with water, and boil the suspension for 3–5 minutes. Place a Petri dish cover over the beaker and hold it at 22° C. for 24–48 hours.

3d day. Examine the scum which has formed on the hay infusion in hanging drop and stained preparations (simple stains, Gram's method). Pour some agar plates and streak in dilutions on two blood plates. Incubate at room temperature and at 37° C.

4th day. Examine the cultures as outlined for *B. subtilis.* Make subcultures and identify the pure cultures (probably *B. mycoides, B. megatherium, B. mesentericus,* etc.), after studying Lawrence, J. S., and Ford, W. W., Aerobic Spore-bearing Non-pathogenic Bacteria. *Jour. Bacteriol.,* 1916, 1, pp. 273–319.

Before the close of the week be sure that all tubes, Petri dishes, etc., infected with anthrax material have been properly sterilized. Keep only the set of tubes under observation. Discard all others. Do not keep any Petri dishes in the desk.

References:

LEAKE, J. P., AND LEDERER, A. Isolation of anthrax bacillus from shaving brushes. *Am. Jour. Pub. Health,* 1919, 9, p. 114.

HAGAN, W. A. The diagnosis of anthrax from putrefying animal tissues. *Jour. Bacteriol.,* 1920, 5, p. 343.

Fifth and Sixth Weeks

XIII. Complete the work on *B. anthracis.*

XV. Microscopic examination of infant's stools—nursling and artificially fed.

XVI. Microscopic examination of normal adult's stool.

XVII. Study of pure cultures of *B. typhosus, B. paratyphosus* A and B, and *B. proteus.*

XVIII. The isolation and identification of members of the colon-typhoid group from infected stool specimens.

XIX. The Widal reaction.

Special Materials Required, Fifth and Sixth Weeks—

Culture Media

Brown cresol purple milk (No. 14).
Dunham's peptone solution (No. 19).
Gelatin (No. 22).
Neutral red broth (Appendix A).
Nitrate solution (No. 52).

Dulcitol, Glucose, Lactose, Maltose, Mannitol, Sucrose — Peptone solution containing Andrade's indicator and small vials to test gas formation (No. 15).

Lead-acetate agar (No. 37).
Peptic digest agar (No. 57).
Peptic digest broth (No. 58).
Potato slabs (No. 61).
Russel's double sugar agar (No. 62).
Veal infusion broth 25–30 c.c. amounts (No. 77).
Endo plates (No. 20).
Eosin methylene blue plates (No. 21).
Brilliant green eosin plates (No. 13).
Brilliant green 1:10,000 dilution.

Stool Specimens from

(*a*) Nursing infants.

(*b*) Artificially fed infants.

(*c*) Normal adult.

Pure Cultures of

(*a*) *B. acidophilus.*

(*b*) *B. typhosus.*

(*c*) *B. paratyphosus* A.

(*d*) *B. paratyphosus* B.

(*e*) *B. proteus.*

Slides showing *B. bifidus.*

Stool, urine, blood cultures containing unknown organisms of the colon-typhoid-paratyphoid group.

Specific antiserums against

B. enteriditis
B. paratyphosus A
B. paratyphosus B
B. typhosus

} Diluted in dropping bottles and in 1:20 dilutions in small flasks.

Standard antigens prepared with the species enumerated above.

Carbolized or formalinized buffered saline; pipettes; agglutination tubes.

Fifth Week

XIII (Completed).

1st day. Examine the cultures inoculated with *B. anthracis* and *B. subtilis* and tabulate the result. Discard the infected tubes and be sure they have been properly sterilized for 2 hours at 15 pounds pressure.

XV. Microscopic Examination of Infant's Stools—Nursling and Artificially Fed.

1st day. Note the difference in the color, consistency and odor of the specimens supplied. Emulsify a small amount (size of a pea) of the stool specimen in saline solution and prepare thin films. Stain with Gram's stain—use Kopeloff and Beerman's modification. What type of organism predominates? Note the differences in the bacterial flora in the specimens furnished. Examine slides showing *B. bifidus.* Pure cultures of *B. acidophilus* will be supplied and should be studied in the manner outlined below. What are the physiological differences between a Gram positive and a Gram negative flora?

XVI. Microscopic Examination of Adult's Stools.

1st day. Prepare slides with normal adult stool specimen in the same way as in XV. Note the different types.

XVII. Study of Pure Cultures of *B. typhosus, B. paratyphosus* A and B, and *B. proteus.*

1st day. Pure cultures of *B. typhosus, B. paratyphosus* A and B, and *B. proteus* will be supplied.

1. Make hanging drop preparations and smears stained with toluidin blue or methylene blue by Gram's method.

2. Transplant a loopful each of *B. typhosus* and *B. paratyphosus* A or *B. paratyphosus* B, and *B. proteus* to 3 peptic digest agar slants respectively. Pour some agar plates for colony study. Incubate at 37° C. for 24 hours.

3. Practice staining of flagella on the culture of *B. proteus* supplied. Use either Zettnow's, Shunk's, or Plimmer and Paine's method.

2d day. 1. Examine the slants and stain by Gram's method to verify purity. If pure inoculate into the following mediums: Dunham's peptone broth, nitrate broth, potato slant, brom cresol purple milk, gelatin, lead-acetate agar, neutral red broth, lactose, glucose, sucrose, maltose, mannitol, dulcitol peptone broth containing small fermentation vials, Russel's double sugar agar.

2. Study the colonies which develop on the agar plates.

3. Cultures grown on eosin methylene blue plates and Endo plates will be supplied. Study the appearance of each type and distinguish between lactose non-fermenting and lactose fermenting organisms.

XVIII. The Isolation and Identification of Members of the Colon-typhoid Group from Infected Stool Specimens.

The specimens used in this work will be supplied. Each member of the class is expected to diagnose one stool specimen and report the results to the instructor within the next two weeks.

Procedure: A and B.—

3d day. A. (*a*) To prepare the following medium students should work in groups of six. The media can be prepared while the specimen is being enriched (step *b*). Two students prepare respectively Endo's plates, eosin methylene blue, or brilliant green plates (Teague's). The concentrated sugar solutions, chemicals, and dyes will be supplied.

If the plates have been cooled sufficiently before they are poured, they will not be too moist to be used immediately. If, however, too much water of condensation has collected on the lids of the Petri dishes the plates should be dried in the incubator for from ½ to 1 hour in the following manner: The lid is removed from the Petri dish and the agar containing portion is placed, with its glass surface uppermost, in the incubator one edge resting on a piece of glass tubing or against the glass surface of the lid of the Petri dish. It is advisable to have a clean sheet of paper under the dish. (*b*) Two loopfuls of the stool specimens to be examined are emulsified in peptic digest broth. The emulsion is incubated for 1 hour. Do not shake. Why?

Remove one loopful of the enriched specimen from the *uppermost* part of the broth to a brilliant green plate or eosin methylene blue plate. With a sterilized bent wire or a glass dalli carefully spread the drop over a narrow strip on the surface of the plate.

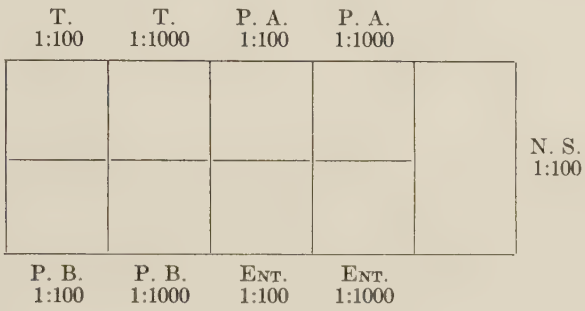
Then flame the dalli, cool—touch an uninoculated portion of the plate several times with the spreader before proceeding to distribute the inoculum over the whole plate. When the dalli is cool recharge by rubbing it over the infected area, then inoculate the remainder of the plate by successive parallel strokes, and finally pass it over the surface of an Endo plate. The method of seeding will be demonstrated. Incubate at 37° C. for 20–24 hours. Plates inoculated in this manner as a rule show well-isolated colonies on both the eosin methylene blue or brilliant green plate and on the Endo plate. It must be remembered that in practical work 3, 4, and occasionally 5 dilution plates are made. In doubtful cases plates are prepared directly from the heavy stool emulsions and from the enriched cultures.

4th day. (*a*) Examine the plates inoculated yesterday. Distinguish between *lactose fermenting* and *lactose non-fermenting* colonies. Plates with colonies of lactose fermenting and non-lactose fermenting organisms will be available for comparison. On the eosin methylene blue plates the colonies of the lactose ferments appear as round, slightly raised, regular, opaque, colonies usually pigmented deep blue but sometimes with a violet tinge. They sometimes show concentric rings on the surface and have a slightly metallic luster. The colonies of the non-lactose fermenting bacteria are round, slightly raised, smooth, grayish and transparent, and have a glassy appearance. They should be distinguished carefully from colonies of streptococci which may develop on such media. The latter are smaller, more elevated, have less luster than the typhoid colonies, and usually have a faint blue tinge. Make an impression preparation as practiced in the exercise on anthrax. (*b*) *Presumptive test for organisms* of the typhoid-paratyphoid group. In order to make a rapid tentative diagnosis it is often advantageous to carry out *slide agglutination* tests with suspicious colonies. When the suspicious colonies on the plate are few in number, only a portion of the colony can be used to carry out the test. It is therefore advisable in such instances to transplant a minute amount of the suspicious colony to a *Russell's double sugar agar slant and a peptic digest agar slant.** For comparison transplant a *B. coli* type to Russell's double sugar agar. If there are many suspicious and well-isolated colonies on the plate the slide agglutination test may be carried out first.

* If transplants are made to peptic digest agar slants early in the morning there will be sufficient growth by the afternoon (6–7 hours) to permit one to make transplants to the differential media and to carry out a macroscopic agglutination test. In this way a diagnosis can be given within 48 hours.

TECHNIC OF THE MACROSCOPIC SLIDE AGGLUTINATION TEST

1. Prepare dilutions (1:100, 1:1000) of known agglutinating serums for *B. typhosus, B. paratyphosus* A, *B. paratyphosus* B, and *B. enteritidis* in Krumwiede's dropping bottles (*Jour. Immunol.,* 1920, 5, p. 155)). Normal serum (1:100) of the same animal species which furnished the immune serum should be at hand.



2. Divide a clean slide into 9 parts and place a small drop of the diluted serum in the designated sections. With a needle emulsify *very evenly* a small amount of the suspected colony, first in the normal serum, then in the highest (1:1000), and finally in the lowest dilution (1:100) of the antiserum. *Never* carry the needle from one type of serum to the other: always flame and use a new inoculum. Observe the slide with the naked eye and with a hand lens and watch for flocculation. Specific agglutination may appear almost at once (within ½–1 minute). Sometimes the clumping is retarded but may be hastened by *gently* warming the slide. Occasionally the organisms do not agglutinate at all. This may be due to several factors. Sometimes strains isolated directly from the body are inagglutinable (why?) and only become agglutinable after several successive transplants on artificial culture media. At times individual colonies from the same strain differ serologically, some may react very slowly, others not at all. Therefore a negative slide agglutination reaction is not conclusive. Colonies apparently typical but not distinctly agglutinable should be fished for further identification by step (*c*).

(*c*) Make transplant to Russell's double sugar agar of colonies giving a positive reaction and one or two lactose fermenters (*B. coli*). Incubate at 37° C. for 24 hours and hold for the following week.

Procedure: B—

3d day. B. A group of students will test the brilliant green enrichment method for the isolation of the paratyphoid-typhoid organisms from feces. This method depends on the selective inhibition of the prevalent types of *B. coli* by brilliant green.

If the stool is solid or semi-solid, a dense emulsion is prepared in sterile salt solution, and from this or from the liquid stool, three tubes of 10 c.c. of peptone water (2 per cent peptone, 0.5 per cent sodium chloride P_H 7.0), containing the following amounts of a 1 in 10,000 watery solution of brilliant green are inoculated:

1	2	3
0.25 c.c.	0.4 c.c.	0.7 c.c.

The 1 in 10,000 solution is made up when required from a stock 1 per cent by the addition of 0.1 c.c. to 9.9 c.c. of sterile water, and the appropriate amounts are added to the peptone tubes.

In general a large loopful of the liquid fecal emulsion is added to the tubes. The tubes are incubated at 37.5° C. and after 12 hours subcultures on eosin methylene blue or brilliant green plates are made from each. Continue isolating the unknown organism according to the outline given under "A."

For the isolation of members of this group from blood and urine see Part IV and Part V.

Sixth Week

XVIII. Colon-Typhoid Group (*Completed*).

1st day. 1. Make a Gram stain of the growth on the Russell's double sugar. What is the value of using the Russell's double sugar medium? If the growth is pure, (*a*) inoculate a set of differential medium with the suspected typhoid or paratyphoid culture and (*b*) another set with a lactose fermenter (*B. coli*). Incubate at 37° C. Observe and record results after 24 hours, 3, and 5, days. When atypical cultures are encountered it may be necessary to continue the observations for 10–30 days. Examine the young cultures for motility. The tests for the reduction of nitrates and for the production of indol should be carried out *after 4 days'* incubation. For details consult table 6.

2. Confirmatory agglutination test. Inoculate a flask containing about 25 c.c. of veal infusion broth with the suspicious colony. Incubate at 37° C. for 18–24 hours.

2d day. Add 0.1 per cent formaldehyde (1.0 c.c. of neutral commercial formalin) to the 25 c.c. veal broth culture planted with the suspicious colony. Place the formalinized suspension for 5 days in the ice box. Then test the sterility by transferring 0.5 c.c. to glucose peptic digest broth and peptic digest agar slant. *Mount pipette in safety cap.* When the culture is sterile compare the density with that of a standard suspension supplied by the laboratory. If necessary dilute it with formalinized buffered saline until the same opacity as the standard has been secured. The standard suspension is always used as a control for the antiserum which will be employed to confirm the biochemical diagnosis of the unknown intestinal organism. For dilution technic, see page 123.

XIX. Macroscopic Agglutination Test. "Widal."

Apparatus Required—

2 1 c.c. pipettes graduated to the tip in 1/10th and 1/100ths.
Safety cap.
Sterilizer with boiling water for pipettes.
Sterile, formalinized buffered saline.
Test tubes.
Agglutination tubes.
Centrifuge tubes.
Beaker for saline.
Suitable test tube racks.

Pasteur pipettes.
Formalinized antigens.
Sterile syringe and two sharp No. 18 gauge needles.
Rubber tourniquet.
Tincture of iodin.
Collodion.
Sterile gauze.

1st day. Each student will aid another in taking a sample of blood for the Widal test. Sterilize needles and syringes at 150° C. for 2 hours. See page 60. The technic of withdrawing blood from the median basilic vein will be demonstrated. Briefly it is as follows:

1. Place a rubber tourniquet (garter elastic with a slip knot) or a few firm turns of a wide muslin bandage above the elbow.

2. Apply alcohol, ether, and tincture of iodin to the skin over the vein. The vein may be rendered more prominent by directing the patient to open and close the hand several times.

3. Mount the syringe with the barrel and needle. Steady the skin over the vein, and insert the needle in the direction of the blood current. By gentle suction aspirate 10 c.c. of blood.

4. Loosen the tourniquet, withdraw the needle quickly, apply a gauze pad, and instruct the patient to hold it with the fingers of the other hand while the phlebotomized arm is bent toward the shoulder. Subfascial hemorrhages may thus be avoided. Seal the wound with a touch of iodinized collodion.

5. Transfer the blood aseptically to a small test tube or preferably centrifuge tube. Rinse the needle and syringe in tap water or 2 per cent "Lysol."

Other methods of obtaining small amounts of blood will be shown, e.g., pricking of the ear lobe and aspiration of the blood in Wright's capsule. *Do not collect blood by puncturing the finger near the base of the nail.* There is danger of subsequent infection. In clinical work the method may be used.

The requisites are: Straight Hagedorn needle, small glass cutting file, gauze, alcohol and ether and a Wright's capsule (2 inch plain sterile glass tube drawn out at the ends to capillary dimensions and sealed, with one of the capillary limbs bent in a semicircle). Cleanse the needle and the patient's ear lobe with alcohol and ether. Break off the tips of both extremities of the capsule, notching first with the file. Puncture the skin by a deep thrust of the needle. When a large drop of blood collects, dip the end of the bent limb of the capsule into it and allow the blood to pass into the capsule by capillary action.

The success of the method depends on getting a continuous free flow of blood. To close the capsule, heat the straight end in the Bunsen flame and seal off the tip. As this end cools, the blood is retracted from the bent limb, which may then be sealed without heating or charring the blood. Both ends must be *completely* sealed. When the blood coagulates in the capsule, the serum can be separated by centrifugalization, the bent limb serving to hang the capsule on the rim of the centrifuge tube. The capsule is opened by filing and breaking it at the bent end, and the serum is drawn off by means of a teat and capillary pipette.

6. *Separation of serum from clot.*—Allow the blood to clot by placing it in the incubator for 30 minutes. Then loosen the clot with the flamed platinum needle. Place the tube back in the incubator for 15 minutes, then in the ice chest for 18 hours. This procedure usually gives a good yield of serum.

2d day. With the aid of a Pasteur pipette mounted with a nipple remove carefully the separated serum; if necessary clear by centrifugalization (15 minutes at 2,000 revolutions). To save test tubes, pipettes, etc., the centrifugalization can be carried out in the presence of the clot. If cotton plugs are used instead of corks secure the free end of the plug by folding and fastening it securely over the lip of the tube with heavy thread or with 2 rubber bands. To the clear serum add a drop of chloroform and keep it in the ice box until needed for the Widal test.

A. Macroscopic Widal Test.

3d or 4th day. Each student determines the titer of his own serum for *B. typhosus, B. paratyphosus* A and B, and *B. coli.* Standard antigens will be supplied. Proceed in the following manner:

MODIFIED DREYER TECHNIC FOR THE DETERMINATION OF THE AGGLUTININ TITER OF BLOOD SERUM

Place in an agglutination rack a series of 10 or more small test tubes ($\frac{3}{8} \times 3$ inches), the number depending on the titer of the serum. Set up 4 such rows. To the first tube in row 1, add 0.4 c.c. of serum plus 3.6 c.c. of saline-dilution 1:10. To each of the next 7 or more tubes add 2 c.c. of saline. To the ninth tube in each row add 0.5 c.c. saline and to the tenth tube 0.5 c.c. normal rabbit serum diluted 1:10. Tubes 9 and 10 serve as controls. Beginning with the first tube transfer (after thorough mixing) 2 c.c. of the diluted serum to the second tube. This gives a dilution of 1:20.

Then continue—from the second tube transfer 2 c.c. to the third and so down through the eighth tube. The eighth tube therefore contains 4 c.c.—discard 2 c.c. In this manner the following geometrical series of dilutions are obtained:

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
1:10	1:20	1:40	1:60	1:160

Tube 9	Tube 7	Tube 8	Tube 9	Tube 10
1:320	1:640	1:1280	Saline control	Normal serum control

With a pipette transfer 0.5 c.c. from each tube in the series to the corresponding tubes in the other three series beginning at the highest dilution (eighth tube) and continuing through to the first tube. Add 0.5 c.c. of standardized *B. typhosus* antigen to the first row, 0.5 c.c. standardized *B. paratyphosus* A antigen to the second, *B. paratyphosus* B to the third, and *B. coli* to the fourth row. Shake the tubes gently. Incubate for 2 hours at 50°–55° C. or for 3 hours at 37° C. and then hold at room temperature for 12–16 hours. The highest dilution showing definite clumping is taken as the end point. Normal serum and saline controls must of course remain turbid. *Keep the remainder of the serum for other tests.*

B. Microscopic Widal Reaction

A group of properly qualified students will demonstrate the microscopic agglutination reactions.

Preliminary dilutions of serum are prepared one-half the strength of the final dilutions (1:10, 1:20, etc.) desired. A loopful of each dilution is placed on separate cover slips, as well as a drop of the diluent. Then at the side of each drop is placed a loop of an 18-hours-old broth culture of *B. typhosus* (if necessary properly diluted with broth). The two drops on each slip are then mixed with the platinum wire. This results in a doubling of each of the blood dilutions. Hollow ground slides, the edges of the concavities having been smeared with vaseline, are warmed and placed over the cover slips so as to seal the drop in the concavity. The seal must be complete or the drops will dry up. The slide is then turned face up and the reaction observed under the No. 6 objective ($\times$ 500 magnification). The slide smay be kept at room temperature, 20° C., or at incubator, 35° C. The same controls as in the macroscopic test are needed. Note carefully the various phases of the reaction. Is there any lysis? In what dilution? When is the microscopic method used?

Seventh Week

XVIII. The identification of the unknown organism belonging to the "colon-typhoid-paratyphoid" group continued.

XX. Identification of organisms belonging to the group of dysentery bacilli.

XXI. Isolation of cholera vibrios from infected stools.

XXII. Water analysis.

Special Materials Required—

Culture media—as specified for the fifth and sixth weeks.
Sterile glass stoppered bottles to collect water samples.
Aronson's media (No. 2).
Blood agar plates (No. 6)).
Standard media for water analysis (No. 38)).
Lactose broth in Smith fermentation tubes (No. 43).
Pure cultures of *B. dysenteriae* Shiga, Flexner, Kruse, E., and Schmitz.
Pure cultures of *V. cholerae.*
Stool specimens infected with organisms belonging to the dysentery group.
Polyvalent dysentery antiserum, Flexner and Shiga types.
Monovalent dysentery antisera.
V. cholerae antiserum.

XVIII. Complete the Identification of the Unknowns belonging to the Colon-Typhoid-Paratyphoid Group.

1st day. Test the sterility of the formalinized veal broth antigen prepared last week by subculturing 0.5 c.c. in glucose peptic digest broth and on a peptic digest agar slant. Incubate 24 hours at 37° C.

2d, 3d, or 4th day. With the formalinized broth antigen prepared, confirm the diagnosis of the unknown by means of the macroscopic agglutination test. Known antisera will be supplied. Use either the same procedure as in the Widal test or in case the antiseras are very potent use the following range of dilutions: 1:20, 1:40, 1:60, 1:80, 1:100, 1:200, 1:400, 1:600, 1:800, 1:1000, 1:2000, 1:4000, 1:6000, 1:8000, 1:10,000. From the original serum prepare a 1 to 10 dilution (1 part serum + 9 parts of saline) and then prepare from this a 1:100 dilution, from this a 1:1000 dilution and from this a 1:10,000 dilution, etc.; the intermediate dilutions are easily prepared thus:

1 to 10 dilution	1 part	1 part	1 part	1 part	
+ saline	1 part	2 parts	3 parts	4 parts	etc.
gives a dilution of	1:20	1:30	1:40	1:50	

Intermediate dilutions are similarly prepared in the hundreds, thousands, etc. In setting up the reaction always add 0.5 c.c. of bacterial suspension to 0.5 c.c. of the serum dilution: *this doubles the dilution. This final dilution is used for recording purposes.* When using this method of preparing the dilutions, rinse the pipettes carefully with saline and dry with blotting paper between each transfer of serum. Hand in a written report on the diagnosis by the end of the week.

XX. Identification of Organisms belonging to the Group of Dysentery Bacilli

1st day. 1. Examine a pure culture of *B. dysenteriae* Flexner by Gram's stain and for motility.

2. Each student will plate a stool specimen containing one of the various types of dysentery bacilli on eosin methylene blue and Endo plates. Proceed in the following manner.

If the stool contains mucus, remove a small flake and wash it in 10 c.c. sterile salt solution. Then place the flake on an eosin methylene blue plate and with a dalli spread over the first, then, without flaming the dalli, to the second, and finally to the third plate. Some students will repeat the same procedure on Endo plate.

If the specimen does not contain mucus plate out a loopful of the liquid stool making 3 dilutions on eosin methylene blue plates as described above. Why are the specimens not enriched before plating? Incubate for from 18 to 24 hours.

2d day. Examine the plates and determine the pathogenic microorganisms in a manner similar to the outline given for the colon-typhoid group. Use the following carbohydrates: lactose, glucose, mannite, maltose, and saccharose. Slide agglutinations should be carried out with polyvalent dysentery antiserum using normal serum as a control. Why?

NOTE: For the final identification of the dysentery bacillus the antigens should be prepared in the following way: Grow the suspicious colony in 10 c.c. plain broth, incubate 24 hours, add 0.1 per cent formalin, place in ice chest for 4–5 days. Test sterility of the antigen before using. Carry out the agglutination test in the same way as previously described for the organisms belonging to the colon-typhoid group. Hold the serum dilutions-antigen mixtures at 37° C. for 18 hours before the final reading is taken.

XXI. Study of *Vibrio cholerae.*

1st day. (*a*) Each student will examine a pure culture of *Vibrio cholerae* by Gram's method, hanging drop preparation, and inoculate Dunham's peptone solution, a gelatin stab, tube of milk, saccharose, and glucose peptone solution.

The pellicle of the peptone solution is examined for motility and by Gram's method. The cholera red test is tried; if negative the procedure is repeated on the third and fourth days. Why is the gelatin liquefied? What other organism can form a pellicle in peptone water?

(*b*) A group of students will isolate *Vibrio cholerae* from a stool specimen.

1. Examine an emulsion from a small flake of fecal mucus (*a*) in hanging drop, (*b*) stained with dilute carbolfuchsin, and (*c*) by Gram's method.

2. Inoculate a tube of peptone water P_H 8.0–8.4. Incubate the peptone water for 4 hours at 37° C. Then from the surface of the enriched culture which for convenience will be called the first enrichment culture transfer (*a*) 1 loopful to the surface of *Aronson's* or *Dieudonne's medium* and streak over the surface in the usual manner. (*b*) 1 loopful to another tube of peptone water = second enrichment. Incubate all tubes and plates for 18 hours.

2d day. 1. (*a*) If suspicious colonies are present (*V. cholerae* on Aronson's medium produces a delicate red colony in contrast to the large colorless colonies of *B. coli.* On a properly prepared Dieudonne's medium *B. coli* is inhibited) make smears and stain with dilute fuchsin. By Gram's method, determine the motility in a hanging drop preparation and carry out slide agglutination tests with suspicious colonies. (*b*) If suspicious colonies are not present make a series of dilution surface plates from the incubated culture of the first enrichment and another series from the second enrichment.

2. Test the 24-hour culture of the first enrichment for the production of indol, by the addition of 3–6 drops of pure concentrated H_2SO_4 to each enrichment. How does this reaction known as the 'cholera red' reaction differ from the Salkowski indol test? Controls should be tested at the same time, as some uninoculated peptone solutions may give positive color tests.

3. Transplant suspicious colonies to agar slants and at the same time replate on Aronson's medium to test for the purity of the culture.

3d or 4th day. Test the pure cultures on gelatine, Loeffler's serum, brom cresol purple milk, Dunham's peptone solution, glucose, lactose, and saccharose broth and on blood agar plates.

XXII. Water Analysis.

A group of students under the supervision of a designated leader conduct a bacteriological examination of different samples of water. Students isolating *Vibrio cholerae* from stools will be excused from this exercise but will be expected to know the *procedure and principles* involved in a sanitary water analysis.

1st day. Obtain the sample in the sterile containers as directed and proceed with the bacteriological analysis. If in any way feasible keep the samples on ice. It is recommended that the following sources should be investigated: laboratory tap water, men's and women's swimming pools, Strawberry Creek close to Stephens Union and close to the Faculty Club, Berkeley sewage, bay water near Oakland or Alameda, or other samples obtained from the State Hygenic Laboratory.

The dilutions from which significant results may probably be anticipated should be ascertained through conference with the instructor. Following are the directions for the examination of a good quality of water, e.g., from the tap. For details of apparatus, materials, and methods see *Standard Methods of Water Analysis* (American Public Health Association, 1917); Prescott, S. C., and Winslow, C. E. A., *Elements of Water Bacteriology* (ed. 4; New York, John Wiley and Sons, 1924).

Collection of sample—

Use clean bottles sterilized by dry heat for at least one hour and a half at 170° C. or in the autoclave at 15 pounds (121° C.) for 30 minutes or longer after the pressure reaches 15 pounds.

Great care must be exercised to have the sample representative of the water tested and to avoid contamination at the time of filling.

Presumptive test—

1. Shake the bottle at least 25 times.

2. Inoculate lactose broth fermentation tubes with 10 c.c., 1 c.c., 0.1 c.c. by means of sterile pipettes. If a high degree of pollution is suspected (as in sewage) carry the dilutions to .00001 c.c. These tubes should have at least four times as much media as added water.

3. Incubate 24 hours at 37° C.

2d day. 4. From the tube showing gas with the least amount of water inoculated streak out one Endo plate.

3d day. 5. Pick red colonies,* if any, to peptic digest agar slants. Incubate at 37° C.

4th day. 6. Stain the transferred colonies by Gram's method. If these prove to be Gram negative, non-sporulating short rods, confirm as members of the colon bacillus group according to the method outlined on page 117. Record the results according to the table outlined below. (See in this connection the Voges-Proskauer test, Appendix A.)

Nature of sample ..... Source .....

QUALITATIVE TEST

Presumptive test..... Lactose broth: 10 c.c..... 1 c.c..... 0.1 c.c.....

Lactose agar plate from.....c.c. Presumptive test.....

Colony	a	b	c	d	
Gram stain	.....	.....	.....	.....	.....
Lactose broth	.....	.....	.....	.....	.....
Gelatin	.....	.....	.....	.....	.....

QUANTITATIVE EXAMINATION OF WATER

1st day. Have ready ten sterile Petri dishes and four sterile tubes. Into each tube place 9 c.c. of sterile water.

1. Shake the sample at least 25 times.

2. To the first tube add 1 c.c. of the water sample.

3. Rinse the pipette therein and transfer 1 c.c. to the second tube, proceeding similarly through all four. One c.c. of each of these contains respectively 0.1, 0.01, 0.001, and 0.0001 c.c. of the original sample.

4. Beginning with the last and proceeding through the first, with a fresh sterile pipette, transfer 1 c.c. from each to the corresponding Petri dish. Make duplicate plates. Also inoculate the fifth Petri dish with 1 c.c. of the original sample. The ninth serves as an uninoculated control, the tenth as a sterile water control.

* If no red colonies are present, the gas in the presumptive test may be due to anaerobes. In certain cases aerobes may even be responsible, having partially or temporarily lost their ability to display lactolytic properties under aerobic conditions. Standard methods therefore provide, in case there are no red colonies on the plate, that at least two colonies be tested for gas formation in lactose broth.

5. To each Petri dish add about 12 c.c. standard agar liquefied and cooled to 40° to 45° C. Rotate to distribute the diluted water uniformly and allow to harden on a level surface. Incubate inverted at 37° C. for 48 hours.

6. After 48 hours' incubation count the colonies on each plate and record the result in a table as follows:

QUANTITATIVE TEST

Plain Agar, 37° C.

Amount Plated	Count per Plate*	Count per c.c.
1		
0.1		
0.01		
0.001		
0.0001		

If discrepancies in the count per cubic centimeter, as estimated on the basis of the different solutions, are found, attempt an explanation in your notes.

Record your conclusions as to the potability of the water tested and state your reasons in your notes.

References:

GENERAL

Bunting, R. W., and Palmerlee, F. The rôle of bacillus acidophilus in dental caries. *Jour. Am. Dental Assoc.*, 1925, 12, p. 381.

Kopeloff, N. Clinical results obtained with B. acidophilus. *Arch. Int. Med.*, 1924, 33, p. 47.

Kendall, A. I. Recent developments in intestinal bacteriology. *Am. Jour. Med. Sci.*, 1918, n.s., 156, p. 157.

Medical Research Committee, London, Report No. 51, 1920. *The Laboratory Diagnosis of Acute Intestinal Infections including the Principles and Practice of the Agglutination Test.*

Rettger, L. F., and Cheplin, H. A. *A Treatise on the Transplantation of the Intestinal Flora with special reference to the Implantation of B. acidophilus.* New Haven, Yale Univ. Press, 1921.

Rettger, L. F., and Kulp, W. L. Comparative study of Lactobacillus acidophilus and Lactobacillus bulgaricus. *Jour. Bacteriol.*, 1924, 9, p. 357.

* Plates having more than 300 or less than 30 should not be counted, except when the number developing from 1 c.c. is less than 30; then this rule should be disregarded and the count recorded as found.

TYPHOID–PARATYPHOID–DYSENTERY GROUP

ANDREWS, F. W., AND INMAN, A. C. *A Study of the Serological Races of the Flexner Group of Dysentery bacilli.* Medical Research Committee, London, Report No. 42, 1919.

DAVIDSON, W. C. A. Bacteriological and clinical consideration of bacillary dysentery in adults and children. *Medicine,* 1922, 1, p. 389.

HECHT-JOHANSEN, A. *Classification of the Strains belonging to the Typhoid-Paratyphoid Group of Bacteria together with a Demonstration of Differences in Avidity.* Copenhagen, Levin and Munksgaard, 1923.

GAY, F. P. *Typhoid Fever Considered as a Problem in Scientific Medicine.* New York, Macmillan, 1918.

WINSLOW, C. E. A., KLIGLER, I. J., AND ROTHENBERG, W. Studies on the classification of the colon-typhoid group of bacteria with special reference to their fermentative reactions. *Jour. Bacteriol.,* 1919, 4, p. 429.

B. PROTEUS

BENGTSON, I. C. The proteus group of organisms. *Jour. Infect. Dis.,* 1919, 24, p. 428.

V. CHOLERA

GREIG, E. D. The results of the bacteriological examination of the stools of 659 cases of cholera at Calcutta. *Indian Jour. Med. Res.,* 1916, 4, pp. 651, 658; 5, pp. 81, 89, 340.

FOOD POISONING

SAVAGE, W. G., AND WHITE, P. B. *An Investigation of the Salmonella Group with special reference to Food Poisoning.* Medical Research Council, London, Special Report Series No. 91, 1925.

WATER

Bacteriological standards for drinking water. Public Health Reports, 1914, 45, p. 2959.

Standard Methods for the Examination of Water and Sewage. Ed. 5; New York, Am. Pub. Health Assoc., 1923.

Eighth Week

XX. Complete the identification of the organisms belonging to the dysentery group.

XXII. Complete the identification of organisms isolated from water.

XXIII. Identification of the organisms belonging to the Brucella group.

XXIV. Examination of members of the Pasteurella group.

XXV. Study of *B. pestis.*

XXVI. Demonstration of animals infected with *B. tularense.*

XXVII. Examination of the organism of swine erysipelas.

XXVIII. Bacteriologic examination of milk and ice cream.

Special Materials Required—

Solution of methylene blue (National Aniline Co.) made by dissolving 1.1 gram of dry dye in 500 c.c. of distilled water.
Standard pipettes calibrated in 0.01 c.c. for determining the bacterial count of milk.
Sterile 9 c.c. saline blanks.
Sterile 99 c.c. saline blanks.
5–6 sterile 1 c.c. pipettes for each student.
Blood agar plates (No. 6).
Glycerin peptic digest agar slant (No. 30).
Standard agar for milk analyses in 100 c.c. amounts (No. 38).
Mediums ordinarily used for the identification of intestinal organisms. In addition 2 per cent glucose Dunham's peptone solution for Voges-Proskauer reaction.
Adonite, Dulcite, Rhamnose, Xylose } Peptone solution containing fermentation vials and Andrade's indicator (No. 15). (The sugars used for the diagnosis of organisms of the Brucella or Pasteurella group should contain serum [No. 16].)
Samples of milk from about 6 different sources and of varying quality.
Pure cultures of *B. alkaligenes.*
Pure cultures of *Br. abortus.*
Pure culture of *Br. melitensis.*
Killed culture of *B. pestis.*
Killed culture of *B. tularense* grown on serum glucose cystin agar.
Guinea pigs infected with *Br. abortus.*
Guinea pigs infected with *B. pestis.*
Pigeons infected with *B. avisepticus.*
Mice infected with swine erysipelas.
Rabbits or rats infected with *B. murisepticum.*

Rabbits and guinea pig infected with *B. tularense.*
B. abortus antiserum.
B. melitensis antiserum.
Formalinized antigen of *Br. abortus* and *Br. melitensis.*

XX. Complete the Identification of the Organisms belonging to the Dysentery Group.

BIOCHEMICAL TESTS

1st day. Observe and record the biochemical tests on *B. dysenteriae.* (See table 6.)

2d day. Identify the variety of *B. dysentery* present in the specimen by means of the macroscopic agglutination tests using the formalinized 24-hour-old broth culture prepared last week as an antigen. Use the modified Dreyer technic in arranging the tests. See page 123. Hand in a diagnosis by the end of the week.

XXII. Complete the Identification of Organisms isolated from the Water.

1st day. Transplant the pure culture isolated last week from water into the following mediums to determine the type of organism present: Brom cresol purple milk, Dunham's peptone solution, gelatin, glucose neutral red broth, 2 per cent glucose peptone solution; glucose, lactose, saccharose and adonite peptone solution with Andrade's indicator and fermentation vials. Incubate cultures at 37° C. for 4–5 days.

4th day. Test for acid production (methyl red), indol, and acetyl methyl carminol (Voges-Proskauer reaction).

XXIII. Identification of the Organisms belonging to the Brucella Group.

Recent studies have established a close relationship between *Brucella melitensis,* the causative organism of Malta or undulant fever, and *Brucella abortus,* the organism responsible for contagious abortion of cattle. Pure cultures of the *Br. melitensis, Br. abortus,* and *B. alkaligenes* will be available. Make hanging drop preparations and stain films by Gram's method and dilute methylene blue. Are the organisms rods or cocci? Prepare subcultures on glycerin peptic agar slants, milk, gelatin, glucose, and lactose carbohydrate broth. Incubate for several days at 37° C.

3d day. Guinea pigs infected with *Br. abortus* will be demonstrated and the significance of the lesions explained by the instructor. Spleen cultures will be prepared and demonstrated. Why are the tubes sealed with sealing wax? The members of the class will study the morphology and cultural characteristics of *Br. abortus* and *Br. melitensis* and compare them with those of *B. alkaligenes.*

Extreme care must be exercised while working with these organisms; laboratory infections are not uncommon.

DIAGNOSTIC AGGLUTINATION TEST

4th day. A group of students will conduct agglutination tests with the remainder of the sera of the members of the class, which were used for the Widal test. A positive serum and standard formalinized antigens will be supplied. The technic is the same as used in the Widal test with the exception that the serum dilutions with the antigen are incubated at 37° C. for 4 hours and kept at room temperature over-night.

Interpret the findings. What is the *Br. melitensis* titer of a normal serum? Which serum dilutions are of diagnostic significance? Can *Br. abortus* and *B. alkaligenes* be used as substitutes for *Br. melitensis* in the agglutination test?

References:

Meyer, K. F., and Shaw, E. B. A comparison of the morphologic, cultural and biochemical characteristics of *B. abortus* and *B. melitensis. Jour. Infect. Dis.*, 1920, 27, p. 173.

Feusier, M. L., and Meyer, K. F. Principles in serologic grouping of *B. abortus* and *B. melitensis. Jour. Infect. Dis.*, 1920, 27, p. 185.

Evans, A. C. Nomenclature of melitensis-abortus group of bacterial organisms. Pub. Health Rep., 1923, 38, p. 1943.

Evans, A. C. Serological classification of *Brucella melitensis* from human, bovine, caprine, porcine, and equine sources. Pub. Health. Rept., 1923, 38, p. 1948.

XXIV. Examination of Members of the Pasteurella Group.

3d day. Pigeons infected with *B. avisepticus* and rabbits or rats injected with *B. murisepticum* will be autopsied. Students will again work in groups and each student will examine one organ.

1. Culture the heart blood on blood agar and deptic digest agar plates and bits of liver, spleen and lung on peptic digest agar.

2. Make smears of the heart blood, liver, spleen and lungs. Stain by Wayson's method with thionin and by Gram's method. Describe the organisms found.

4th day. Make Gram and Wayson stains of the colonies which develop from the organs cultured yesterday. Transplant the Gram negative bipolar staining organism to an agar slant. Incubate 24 hours. After pure culture is obtained plant in Hiss serum sugars for identification.

Reference:

BESEMER, A. M. A study of five members (or so-called species) of the septicemia hemorrhagica (Pasteurella) group of organisms, etc. *Jour. Bacteriol.*, 1917, 2, p. 177.

XXV. Study of *B. pestis.*

4th day. Plague infections in man, rats, and ground squirrels are of considerable interest to the physician in California. Because of the great danger in working with this organism, cultures cannot be studied in the class.

The instructor will autopsy a guinea pig and a rat showing acute or chronic plague lesions. He will prepare smears from the organs and fix them in bichloride or formalin solutions. The student will stain them by Wayson's and by Gram's method.

Sterilized cultures on broth, on 3 per cent salt agar and on gentian-violet agar will be available for supplementary study. Films should be stained with methylene blue by Gram's method and with thionin.

Sections showing *B. pestis* in tissues will be demonstrated.

How can *B. pestis* be distinguished from *Pasteurella murisepticum* and *B. enteritidis*?

Reference:

D'AUNOY, R. Studies on Bacillus pestis. *Jour. Infect. Dis.*, 1923, 33, pp. 391–415.

XXVI. Demonstration of Animals infected with *B. tularense.*

Recent studies by Francis emphasize the great importance of *B. tularense* infections in California and other western states.

A rabbit and a guinea pig infected with this organism will be autopsied by the instructor. Killed cultures of *B. tularense* on serum glucose cystin agar will be furnished for microscopic study of the organism. The bacterium may not be studied by students, as laboratory infections are frequent.

Reference:

FRANCIS, E. Tularemia. *Jour. Am. Med. Assoc.*, 1925, 84, p. 1243.

XXVII. Examination of the Organism of Swine Erysipelas.

3d day. Mice previously infected with *B. erysipelatis suis* will be autopsied by assigned groups of students. Autopsy and prepare smears and cultures as outlined for the mouse infected with anthrax. One student of each group will prepare cultures on peptic digest agar plates. Prepare smears and stain by Gram's method. The organism is Gram positive.

4th day. If the cultures are pure make transplants to gelatin stab, peptone solution, solidified blood serum, milk, lactose, glucose, saccharose, serum water. Incubate for 5 days; test for indol and read the carbohydrate reactions.

Reference:

CREECH, G. T. The bacillus of swine erysipelas isolated from urticarial lesions of swine in the United States. *Jour. Am. Vet. Med. Assn.*, 1921, 12, pp. 139–150.

XXVIII. Bacteriologic Examination of Milk and Ice Cream.

1st day. Each student will conduct a bacterial examination of one specimen of milk or ice cream which will be furnished. Use the reductase and the microscopic count method to confirm the plate counts. Discuss and interpret the results in the notebook. Proceed in the following manner:

1. Melt flask of agar.
2. Mix the sample of milk by tilting the bottle 25 times.
3. *B. welchii* test.

Flame the lip of the bottle and fill a sterile test tube about half full with the milk sample. Place the tube in a water bath maintained at 80° C. for 1 hour. Then allow the tube to cool and incubate at 37° C. for 24–48 hours.

4. Pour about 50 c.c. of the sample into a sterile bottle for bacteriologic work.
5. Reductase test.

REAGENT—

Prepare a stock solution of methylene blue (National Aniline Company) by dissolving 1.1 gram of the dry dye in 500 c.c. of distilled water.

Shortly before use, 1 c.c. of the stock solution is diluted with 39 c.c. of distilled water; heat it to 100° C. for a few minutes.

MAKING THE TEST—

Clean sterile test tubes; a 10 c.c. and a 1 c.c. pipette are needed.

Place 10 c.c. of milk in a test tube and then add 1 c.c. of the dye solution (1:20,000 methylene blue). Place the tube in a water bath at 37° C. and observe at regular intervals.

The results may be classed as follows:

CLASS 1. Good milk, not decolorized in 5½ hours, containing, as a rule, less than ½ million bacteria per cubic centimeter.

CLASS 2. Milk of fair average quality, decolorized in less than 5½ hours but not less than 2 hours, containing, as a rule, ½ to 4 million bacteria per cubic centimeter.

CLASS 3. Bad milk, decolorized in less than 2 hours, but not less than 20 minutes, containing as a rule, 4 to 20 million bacteria per cubic centimeter.

CLASS 4. Very bad milk, decolorized in 20 minutes or less, containing, as a rule, over 20 million bacteria per cubic centimeter.

6. Bacterial count.

A. Plate count method.

(*a*) Label Petri dishes (clay top dishes may be used) 1:100, 1:1000, 1:10,000, etc., and control, in duplicate, together with the number of the sample and the date.

(*b*) With first sterile 1 c.c. pipette.

(*aa*) Put 1 c.c. milk into 99 c.c. water blank. Rinse back and forth 3–4 times. Shake 25 times = 1/100 dilution.

(*bb*) With second pipette transfer 1 c.c. quantitites of 1:100 dilution into each of two Petri dishes labeled accordingly. NOTE: Before plating each dilution shake bottle and rinse the fresh sterile pipette by drawing dilution to be plated back and forth several times. 1 c.c. of 1:100 dilution into 9 c.c. water blank = 1/1000 dilution, rinse back and forth 3–4 times, shake bottle 25 times.

(*cc*) With a third pipette transfer 1 c.c. quantities of 1:1000 dilution into each of two Petri dishes labeled accordingly. 1 c.c. of 1:1000 dilution into 9 c.c. water blank = 1:10,000 dilution, rinse back and forth 3–4 times, shake bottle 25 times.

(*dd*) With a fourth pipette transfer 1 c.c. quantities of 1:10,000 dilution into each of two Petri dishes labeled accordingly. Continue making dilutions by plating according to the source of the specimen.

7. Cool agar to 45° C. Flame lip of tube and pour about 15 c.c. agar into each dilution plate. Lift cover only enough to permit the pouring of the plates.

8. Revolve plate slowly and gently—do not tilt. When the agar has hardened invert the plates and incubate for 48 hours at 37° C.

3d day. Count the colonies that have developed on each dilution plate after 48 hours incubation. Plates on which more than 300 colonies or less than 30 colonies have developed are not suitable for quantitative work.

MICROSCOPIC COUNT (BREED OFFICIAL METHOD)

1st day. 1/100 c.c. milk or cream is spread with a needle over an area of a square centimeter. The film is allowed to dry. The slides are fixed in methyl alcohol for one minute or more and the fat extracted in ether, then drained and dried again. Stain with Loeffler's methylene blue to desired intensity. If necessary decolorize in alcohol. Smears may be kept in the slide box for a day or two after they have been fixed and stained, before the actual count is made.

Standardization of the Microscope

The microscope used must be so adjusted that each field covers a certain known fraction of the area of a square centimeter. Place the stage micrometer on the stage of the microscope, and by selection of oculars or by adjustment of the draw tube, or both, bring the diameter of the whole microscopic field to 0.205 mm. When so adjusted, each field of the microscope covers an area of approximately 1/3000 cm^2. This means that the dried milk solids from 1/300,000 part of a c.c. of milk are visible in each field of the microscope. Therefore if the bacteria in one field only are counted, the number found should be multiplied by 300,000 to give the estimated number of bacteria per c.c. In practice, however, more than a single field is examined so that the number used for multiplication is smaller than this.

Using the oil-immersion lens, count organisms in field of known area. To make this comparable to agar plate count, groups of organisms of the same kind are counted as one. Count from ten to fifty fields in this manner and determine average. Then if the area of each field counted is, for example, 1/3000 of a square centimeter, the final count in 1 c.c. would be x (the average of each field) multiplied by 3000, multiplied by 100 (the amount of milk taken).

Reference:

Standard Methods of Milk Analysis. Ed. 4; Am. Pub. Health Assoc., 1923.

Ninth Week

XXIII. Complete the identification of the organisms belonging to the *Brucella* group.

XXIX. Methods of staining tubercle bacilli in infected specimens.

XXX. Demonstration of tuberculous rabbits and guinea pigs.

XXXI. Methods of isolating tubercle bacilli.

XXXII. Demonstration of tubercle bacilli by animal inoculation.

XXXIII. Demonstration of *B. smegma, B. leprae,* and other acid-fast bacteria.

XXXIV. Study of *C. diphtheriae* and diphtheroids.

Special Materials Required—

Antiformin and 3 per cent sodium hydroxide solution.
Large centrifuge tubes, 50 c.c. or 100 c.c. capacity.
Glycerin veal agar "deeps" (No. 32)).
Loeffler's serum slants (No. 48)).
Greenspoon's medium in slants (No. 34).
Petroff's medium (No. 60).
Acetic acid toluidin blue.
Greenthal's Kresylecht stain.
10 per cent sodium bisulfite.
Sterile sheep's blood.
Sputum specimens from active cases of tuberculosis.
Autopsy specimens with lesions of tuberculosis.
Emulsions of pure and of mixed cultures of
(1) *C. diphtheriae.*
(2) *Staphylococcus.*
(3) *Streptococcus.*
Normal guinea pigs.

XXIII. Completion of the Identification of the Organisms belonging to the *Brucella* Group.

3d day. Read the biochemical reactions produced in the tubes inoculated with *Br. melitensis, Br. abortus,* and *B. alkaligenes.* Record the results in the notebook.

XXIX. Methods of staining the Tubercle Bacillus.

1st day. A. In Sputum. Specimens of sputum containing tubercle bacilli will be available for staining. Pour the sputum into a Petri dish and with the flamed platinum wire pick out small white or caseous particles. If none are present prepare smears from the yellowish portions of the sputum. The smears are prepared on new, clean slides and are examined microscopically in stained preparations by the following methods:

(1) Gram's stain.
(2) Ziehl-Neelsen acid-fast stain.
(3) Konrich's stain.

Always use fresh filter paper for blotting the different slides; why?

B. Methods of demonstrating Tubercle Bacilli in Urine

(*a*) The catheterized urine is centrifuged. It is always advisable to centrifuge a large specimen of urine, at least 50 to 100 c.c. The specimen should be freed from uric acid by gently warming before treating it. Furthermore, it should be neutral; the alkalinity can be corrected by adding a few drops of dilute acetic acid. The sediment is spread in thin films and stained by Konrich's method and by Pappenheim's method to differentiate between the tubercle and the smegma bacillus. The sediment may also be used for the inoculation of guinea pigs (see XXXII).

(*b*) Alcoholic concentration method. Mix equal parts of urine and 95 per cent alcohol. Centrifuge at high speed for 30 minutes. Remove the supernatant fluid and make smears of the sediment. In preparing the smears it may be necessary to add a drop of serum or egg albumen to the slide.

C. Demonstration of Tubercle Bacilli in Feces

(*a*) The feces are searched for mucous particles or pus, and films prepared from this material. If none are found large specimens of stool are treated by the antiformin method (p. 141). The sediment is neutralized with dilute acetic acid and examined microscopically in the usual way. Tubercle bacilli swallowed by the patient may appear in the feces.

(*b*) Mix equal quantities of stool and distilled water or saline. Centrifugalize at low speed to remove the heavy particles. Remove the supernatant fluid and treat with 95 per cent alcohol in the same manner as described under B (*b*).

Pus specimens are examined in a manner similar to that described for the detection of tubercle bacilli in the sputum.

XXX. Demonstration of Tuberculous Rabbits and Guinea Pigs.

2d day. A group of students will autopsy and the instructor will demonstrate to the class:

(*a*) A guinea pig inoculated with human sputum.

(*b*) A rabbit inoculated with the same human sputum.

(*c*) A guinea pig inoculated with bovine tuberculous material.

(*d*) A rabbit inoculated with bovine tuberculous material.

The inoculations have been made during the first or second week of the course.

Pus and scrapings from the lesions should be stained by Gram's, Ziehl-Neelsen's, and Konrich's methods.

XXXI. Methods of Isolating Tubercle Bacilli.

Students working in groups will concentrate sputum specimens. (For concentration of the tubercle bacilli see below, Methods 1 and 2. The ligroin method as described in Park and Williams, ed. 8, p. 449, can be tried.) Several members of the group will attempt to isolate the tubercle bacillus, others will inoculate guinea pigs subcutaneously or intraperitoneally.

CULTURAL ISOLATION OF THE TUBERCLE BACILLUS

To free the contaminated specimen of extraneous organisms it is treated by one of the following methods:

Method 1. Uhlenhuth and Xylander's antiformin method.* Mix equal quantities of sputum and 30 per cent antiformin in a large centrifuge tube (100 c.c. capacity is preferable). Mix thoroughly with a sterile pipette. Place in the incubator for 1–2 hours. Centrifugalize at high speed for 30 minutes. Decant the supernatant fluid. Wash the sediment 2 times with sterile saline. After the last centrifugalization, add about 1 c.c. of saline to the sediment.

Method 2. Petroff's method.† Mix equal parts of sputum and 3 per cent NaOH. Place in the incubator at 37° C. for 30 minutes. Neutralize with normal hydrochloric acid. Centrifugalize for 30 minutes and then decant the supernatant fluid, then add 1–2 c.c. of sterile saline.

* *Arb.* a. d. K. Gesundheitsamt, 1909, 32, part 1.

† A new and rapid method for the isolation and cultivation of tubercle bacilli directly from the sputum and feces. *Jour. Exper. Med.,* 1915, 21, p. 38.

Make stained preparations to ascertain the approximate concentration of organisms in the sediment and then plant 2–3 loopfuls to Petroff's medium. The Petroff's medium is incubated in a tilted position for 2–3 days, then the top of the cotton plug is dipped in hot paraffin to prevent the drying out of the medium. The cultures are kept in the incubator since colonies may not become visible for 12 or more days. If the concentration of the specimens is carried out carefully the method gives very good results. Stain preparations of the growth by Gram's method and by the various acid-fast methods. How do young cultures react to the acid-fast stain? How do they differ from old cultures? Why is the method successful in isolating the tubercle bacillus? Why should it be used? Why are new slides used for sputum examinations?

XXXII. Demonstration of the Tubercle Bacillus by Animal Inoculation.

Inoculate the remainder of the suspension from XXXI subcutaneously in the region of the inguinal lymph-nodes of a normal guinea pig.

Careful records must be kept of the general condition of the guinea pigs. The weight should be taken and recorded and the regional lymph-nodes examined at least twice weekly. If the animal does not show any sign of infection (loss of weight, enlargement of regional lymph-nodes) at the end of 4 weeks,* test the animal intracutaneously with 0.2 c.c. of a 1:10 dilution of O. T. tuberculin. Observe reactions after 24 and again after 48 hours.

Confirm a positive skin reaction by inoculating the infected animal intraperitoneally with 1 c.c. of O. T. tuberculin. Use a normal animal as a control. If the skin reaction is negative wait until the end of 6 or 7 weeks and repeat the test. Perform an autopsy and write a detailed report.

A number of students will stain sections of tuberculous lesions and demonstrate the bacilli in the tissues. Follow the method outlined on page 78.

* In practical work at least two guinea pigs are inoculated with the same specimen. They should not be discarded for at least 3 months.

DIFFERENCES BETWEEN THE HUMAN AND THE BOVINE TYPE OF TUBERCLE BACILLUS

Occasionally it may be desirable to establish the type of tubercle bacillus responsible for the infection. Cultures should be obtained, since the animal tests can be more readily controlled. In comparison to the human type, the bovine type grow poorly on plain egg medium; glycerin egg medium should not be used since it restrains the growth and is frequently responsible for failures. On the other hand, the human type grows readily and abundantly on glycerin egg media and is readily transplanted to glycerine potato or glycerine broth. The bovine type is exceedingly *virulent for rabbits;* the human only slightly so. As a rule 10 mg. of culture or the ground and stained tissues are inoculated intravenously into rabbits. If the rabbit survives 40 to 50 days and on autopsy shows only lesions in the lungs or kidneys or both, the strain is of the human type. With the bovine type the rabbit will usually die before or about this time; a progressive generalized tuberculosis will be found.

XXXIII. Demonstration of *B. smegma, B. leprae,* and other acid-fast Bacteria.

2d day. 1. Stain tissue films of *B. leprae* in the same manner. (Smears will be supplied.) For comparison, stain smears of a culture of *B. smegmatis* by Gram's method, and, in order to determine the acid-fast properties of the organism, use merely 1 per cent HCl to decolorize the film, while on another film employ 3 per cent HCl-alcohol. How does the organism react in its acid-fast properties? What is the significance of these findings? Which organism is acid- and alcohol-fast? Sections of leprous lesions will be demonstrated.

2. Prepare thin films from cattle manure and the slimy deposits on water spigots, stain by Konrich's method and examine the smears carefully for the presence of acid-fast bacteria.

What is the significance of acid-fast bacteria in tap water? What errors have been committed by neglecting the preparation of control smears?

In order to distinguish the tubercle bacillus from common acid-fast bacilli it is always advisable to inoculate animals (guinea pigs) and to attempt the cultivation of the organisms.

3. Preparation of blood agar plates: Prepare blood agar plates by the method described in detail in Appendix B. NOTE: Use the agar prepared during the third week. CAUTION: Avoid unnecessary confusion in the laboratory. To prevent air currents, close all windows. Wash off the desks with 5 per cent lysol solution before beginning the work. Be sure that the cotton plug fits securely in the mouth piece of the pipette. The agar should be thoroughly melted and cooled uniformly and sufficiently (to 50° C.) before the blood is added. If these precautions are taken little difficulty will be encountered in preparing satisfactory plates in a large and dusty laboratory. Incubate the blood agar tubes and plates prepared for 24 hours before using them, then place in the ice box until needed.

XXXIV. Laboratory Diagnosis of *C. diphtheriae.*

A. Examination of Pure and Mixed Cultures—Throat Swabs

Since the laboratory diagnosis of diphtheria depends on the ability of the observer to recognize *C. diphtheriae* in the presence of other organisms found in the mouth cavity and the respiratory tract, emulsions of pure and of mixed cultures of some of the commoner types liable to be encountered in such specimens as *M. catarrhalis, Streptococcus, Staphylococcus,* will be available for microscopic examination.

3d day. 1. Stain the emulsions supplied by means of:

(*a*) Gram's method.

(*b*) Loeffler's methylene blue.

(*c*) Acetic toluidin blue solution.

(*d*) Greenthal's stain.

2. Make dilutions of the mixed suspension on the surface of a blood agar plate and a 5 per cent glycerin veal agar plate. Incubate at 37° C. for from 18 to 24 hours.

3. Make transplants of the mixed suspension to Greenspoon's medium. Incubate at 37° C. for from 18 to 24 hours.
Incubate at 37° C. for from 18 to 24 hours.

4th day. 1. Examine the blood agar and the glycerin veal agar plates inoculated yesterday. Study (*a*) the macroscopic appearance of the colonies, (*b*) the changes produced by the organism on the blood medium, (*c*) the appearance of the colonies under low power. Describe the differences and the colony characteristics of each tube. NOTE: On glycerine veal agar the colonies of *C. diphtheriae* appear as small slightly raised greyish granular colonies. Under low power the granular structure is very characteristic and the serrated appearance of the edges of the colony may help in the differentiation.

2. Stain numerous colonies with acetic acid toluidin blue to familiarize yourself with the types present.

3. If isolated colonies of *C. diphtheriae* are present pick and transplant to Loeffler's serum slants.

4. Transplant a *Staphylococcus aureus* colony to a plain agar slant. Incubate at 37° C. for 24 hours. This culture will be used in the succeeding exercises to study the biochemical activities of the *Staphylococcus* group.

5. Stain smears of the growth on Greenspoon's medium by the methods ordinarily used in diagnosing *C. diphtheriae* (methylene blue, Gram's stain, and Greenthal's stain).

XXXIV. B. The Virulence of *C. diphtheriae* in Field Specimens as determined by the Intracutaneous Test.*

4th day. 1. Preparation of the guinea pig.

(*a*) Two white or buff colored guinea pigs weighing from 300 to 400 grams are weighed and marked.

(*b*) An area about 5 cm. in width and extending along each side of the animal from the fore to the hind legs is carefully shaven or the major portion of the hair is clipped and the remainder removed by means of a depilatory barium sulfide. The animals are then ready for use. Each set of 2 is kept in cages in the animal room until the next period.

* Next week each student will determine the virulence of one of the throat cultures diagnosed as positive by the microscopic test. To facilitate the work which is outlined for the next period it is advisable that the animals be prepared in advance. Two guinea pigs are needed, one test animal and one control. Non-pigmented normal guinea pigs weighing from 300 to 400 grams should be used. About 6 tests may be carried out on each set of pigs. Students will therefore work in groups of 6.

Tenth Week

XXXIV. A. Biochemical activities of *C. diphtheriae.*

XXXIV. B. Laboratory diagnosis of diphtheria.

XXXV. Isolation and identification of organisms belonging to the *Staphylococcus* group.

XXXVI. Isolation and identification of organisms belonging to the *Streptococcus* group—throat flora.

Special Materials Required—

Diphtheria antitoxin.
Diphtheria toxin for the Schick test (M.L.D. must be known.)
Saline with 0.5 per cent carbolic acid.
Sterile swabs for taking nose and throat cultures.
Sterile tongue depressors.
Avery's blood broth (No. 4).
Bile (sterile beef or rabbit).
Blood agar plates (No. 6).
Blood agar slants (No. 6).
Brom cresol purple milk (No. 14).
5 per cent glycerin veal agar "deeps" (No. 32).
Greenspoon's medium in slants (No. 34).
Loeffler's serum in slants (No. 48).
Dextrin, Dextrose, Galactose, Glycerol, Inulin, Lactose, Mannitol, Raffinose, Saccharose, Salicin } Hiss serum water + 1 per cent Andrade's indicator (No. 16).
Veal infusion broth.
16–24-hour-old field cultures from suspected cases of diphtheria.

XXXIV. A. Biochemical Activities of *C. diphtheriae.*

1st day. Stain the cultures from the isolated colonies grown on Loeffler's serum to determine their purity.

2. If pure, transplant to the following media: veal infusion broth, brom cresol purple milk, dextrin, dextrose, saccharose, etc., in Hiss serum water with Andrade's indicator. Incubate at 37° C. and examine after 24 hours and again after 3 and 5 days' incubation.

TABLE 7

FERMENTATION REACTIONS* OF CERTAIN SPECIES OF THE PROPOSED GENUS CORYNEBACTERIUM

Group	Glucose	Maltose	Galactose	Saccharose	Lactose	Dextrin	Mannite	Remarks
Virulent C. diphtheriae	+	+	+	−	−	+	−	C. pseudotuberculosis ovis [C. ovis]† according to the M. R. C. gives sugar reactions similar to C. diphtheriae. The organism produces an exotoxin fatal to guinea pigs which is not neutralized by diphtheria antitoxin.
Non-virulent C. diphtheriae	+	+	+	−	−	+	−	
Diphtheroids; group:								
I	+	−	+	+	−	−	−	
II	+	−	−	+	−	−	−	According to the M. R. C. organisms of the group fermenting glucose and saccharose are probably C. xerosis.
III	+	+	−	−	−	−	−	
IV	+	±	−	−	−	−	−	
V	+	+	+	−	−	−	−	
VI	+	+	+	+	+	−	−	
VII	+	+	−	+	−	−	−	According to the M. R. C., C. pseudotuberculosis murium (Kutscher) [C. murium] gives these reactions.
VIII	+	+	+	+	−	−	−	
IX	+	+	+	+	+	+	−	
X	+	−	+	+	−	−	+	
XI	−	−	−	−	−	−	−	Probably C. hofmannii.

* The fermentation reactions are taken from the report of the Medical Research Council: *Diphtheria. Its Bacteriology, Pathology and Immunology*, by Andrews *et al.*, London, 1923.

The organisms should be grown in Hiss serum water media containing 1 per cent Andrade's indicator and 1 per cent of the test substance.

† + indicates the production of acid, − indicates failure to ferment the test substance. Gas is not produced by members of this group. The more common name is given first. The name given in brackets [ ] is the one proposed by the Society of American Bacteriologists—Bergey's *Manual*.

XXXIV. B. Laboratory Diagnosis of *B. diphtheriae.*

1st day. Sixteen to twenty-hour field cultures grown on Loeffler's serum or on Greenspoon's medium will be given to each student. At least 2 or 3 diphtheria positive and one or two diphtheria negative cultures will be supplied. Proceed in the following manner:

(*a*) Make films and stain:
(1) By Gram's method.
(2) With methylene blue.
(3) With acetic acid toluidin blue.
(4) With Greenthal's Kresylecht stain.

(*b*) From the microscopic findings, determine which cultures are positive.

(*c*) Determine the virulence of one of the positive cultures in the following manner:

(*aa*) Inoculate one of the guinea pigs, prepared last week, intraperitoneally with 500 units of diphtheria antitoxin. This animal serves as the control.

(*bb*) Emulsify the growth of the culture to be tested in 2.5–5.0 c.c. of saline (depending on the proportion of bacilli found in the culture). This suspension should have the approximate turbidity of a standard typhoid antigen.

(*cc*) Carefully fill a sterile syringe with about 0.5 c.c. of the emulsion. Protect the tip of the needle after the syringe has been filled with a small wad of cotton soaked in lysol solution. Inoculate 0.1 c.c.—0.2 c.c. of the suspension intracutaneously into the shaven skin of each of the 2 guinea pigs. Three inoculations can easily be made on each side. Space them so that they are as far apart as possible. The site of inoculation on the control pig should correspond to that on the test pig. The second guinea pig should receive about 150 units of diphtheria antitoxin 5–6 hours after the inoculations are made. This is done to protect the animal against a fatal intoxication. It does not interfere with the skin reactions. Examine the site of injection daily for 1 week. Explain results.

Virulent field cultures produce in 24 hours redness and induration followed on the second or third day by a necrosis which manifests itself by a white area with an extending purple center terminating in sloughing of the epidermis and crust formation.

TABLE 8

Biochemical Reactions of the Most Important Genera of the Tribe Streptococceae

Name of organism	Gelatin	Reduction of nitrites to nitrates	Reaction in milk	Mannite	Lactose	Salicin	Saccharose	Inulin	Raffinose	Remarks
Staphylococcus aureus	+[1]	+	A coag.	+	+	−	+	−	−	Growth on potato; heavy, glistening; orange pigment.
Staphylococcus citreus	+	+	A slow coag.	+	+	−	+	−	−	Growth on potato; thin, at first grayish streak, becoming citron yellow.
Staphylococcus epidermides	+	±	A	−	+	−	+	−	−	Growth on potato; white, not heavy.
Staphylococcus albus	+	±	A coag.	+	+	−	+	−	−	Growth on potato; heavy, glistening and white.
Staphylococcus pharyngis	+	+	A coag.	+	+	+	+	−	+	Growth on potato; heavy, smooth, slimy and white.
Micrococcus tetragenous	−	−	Sl. A slow coag.							Growth on potato; viscid, white.
Diplococcus pneumoniae	−	−	A coag. may be delayed or negative	±	Usually + Occasionally −	±	+	±	Usually + Occasionally −	All members of the group are bile soluble. The group is divided into four serological groups. See page 158.

[1] + Indicates the liquefaction of gelatin, reduction of nitrates to nitrites, the production of acid in carbohydrate media (gas is not formed). − indicates failure to produce these effects. A indicates the production of acid in milk. Coag. indicates the coagulation of milk. ± indicates a variable reaction. In the fermentation tests, the more common reaction is given first.

TABLE 8—(*Continued*)

BIOCHEMICAL REACTIONS OF THE MOST IMPORTANT GENERA OF THE TRIBE STREPTOCOCCEAE

Name of organism	Gelatin	Reduction of nitrites to nitrates	Reaction in milk	Mannite	Lactose	Salicin	Saccharose	Inulin	Raffinose	Remarks
Streptococci[2] Alpha type—produce a zone of green discoloration and zones of hemolysis on blood agar plates.										
S. mitis:										NOTE: Brown has described a type designated alpha prime which, in so far as the reaction on horse or rabbit blood agar is concerned, may be confused with the beta type. This type can be distinguished from both the alpha and beta types in pour plates. The zone around the deep colonies of alpha prime type appear somewhat hazy. Under the microscope a few corpuscles are seen to remain throughout the hemolyzed zone, but they are most numerous near the colony. Surface colonies resemble the beta type because the thin collection of corpuscles near the colony is completely obscured by the over-growing surface colony. All streptococci are insoluble in bile.
1.1 [3]			A coag.	−	+	+	+	−	−	
1.2			A coag.	−	+	+	+	−	+	
1.5			A coag.	−	+	+	−	−	−	
S. salivarius:										
2.1			A coag. ±	−	+	−	+	−	−	
2.2			A coag. ±	−	+	−	+	−	+	
S. equinus:										
3.1			−	−	−	+	+	−	−	
3.2			−	−	−	+	+	−	+	
S. fecalis:										
5.1			A coag.	+	+	+	+	−	−	
5.2			A coag.	+	+	+	+	−	+	
5.4			A coag.	+	+	+	+	+	+	
5.5			A coag.	+	+	+	−	−	−	

[2] The classification given is that of Brown. For the sake of brevity, only the more frequent combinations of fermentation reactions are given in detail. For further classification of streptococci, the monograph of Brown should be consulted (Monographs, Rockefeller Institute, 1919, No. 9).

[3] The numbers indicate the formula of the fermentation reactions as given by Brown. They are convenient for the designation of a strain when fermentation tests are used as a basis of classification.

TABLE 8—(*Concluded*)

BIOCHEMICAL REACTIONS OF THE MOST IMPORTANT GENERA OF THE TRIBE STREPTOCOCCEAE

Name of organism	Gelatin	Reduction of nitrites to nitrates	Reaction in milk	Mannite	Lactose	Salicin	Saccharose	Inulin	Raffinose	Remarks
Streptococci, Beta type—produce a clear zone of hemolysis on blood agar plates.										
S. pyogenes (Rosenbach): 1.1	−		A coag. ±	−	+	+	+	−	−	Serologically, this group can be subdivided into at least 8 subgroups. The serological classification does not coincide with the classification based on carbohydrate fermentation tests. Bergey gives the following serological classification:
S. anginosus (Andrews and Horder): 1.1	−		A coag. ±	−	+	−	+	−	−	1. S. pyogenes (Rosenbach) also called S. erysipelatos (Fehleisen). This seems to be a homogeneous group.
S. equi (Schütz): 3.1	—		coag. ±	−	−	+	+	−	−	2. S. scarlatinae. One group of S. scarlatinae is mannite negative, the other, probably a smaller group, is mannite positive, but serologically, the two groups are identical.
S. subacidus (Holman): 4.1	−		coag. ±	−	−	−	+	−	−	3. S. mixtos—probably the same as S. infrequens (Holman), S. hemolyticus II (Holman).
S. infrequens (Holman): 5.1	−		A coag. ±	+	+	+	+	−	−	4. S. equi (Schütz)—the fermentation reactions given in Bergey's manual do not correspond to those given by Brown or Holman.
5.5	−		A coag. ±	+	+	+	−	−	−	However, Holman's type ,designated S. equi, gives the same fermentation reactions as Brown's type.
S. alactosus (Smith & Brown): 7.1	−		coag. ±	+	−	+	+	−	−	5. S. mastiditis (Guillebau). 6. S. cuniculi (Roos). 7. S. felini (Bayne-Jones and Rivers). 8. S. stenos.
Streptococci, Gamma type—produce no discoloration on blood agar plates.										The fermentation reactions of this group have not been determined. It is best to report this type as Gamma type streptococci and merely indicate the fermentation reactions.

The animals protected with antitoxin develop redness and induration of less diameter than the unprotected animals. The control pigs may show as much edema and discoloration as the normal but there is no necrosis or sloughing even when the contaminating organisms cause abscess formation. In case an animal should die, perform an autopsy with complete tissue cultures and determine the cause of death.

Isolation of Diphtheroids from the Conjunctival Sac or Nasal Passages

2d day. The method of taking throat and nasal swabs, etc., will be demonstrated. Take a swab from the upper nasal passage or from the conjunctival sac and smear over a Loeffler's slant. The swab may be broken off and left in the tube. Incubate 24 hours.

3d day. Make Gram stain and toluidin blue preparations. If diphtheroids are present isolate the strain in a manner similar to that given for *C. diphtheriae.*

4th day. Continue the isolation and the study of the cultural characteristics of diphtheroids.

During the third and fourth days the members of the class will submit to the *Schick test.* Submit on the fourth day a diagnosis of the throat swabs examined at the beginning of the week.

References:

Havens, L. C., and Powell, H. M. The use of the original diagnostic culture for the determination of the virulence of the diphtheria bacilli. *Am. Jour. Hyg.*, 1922, p. 234.

Force, J. N., and Beattie, M. I. Intracutaneous test for the virulence of diphtheria bacilli. *Am. Jour. Hyg.*, 1922, 2, p. 490.

Andrewes, Bulloch, Douglas, Dreyer, Fildes, Ledingham, Wolf. *Diphtheria, Its Bacteriology, Pathology and Immunology.* Medical Research Council, London, 1923.

XXXV. Isolation and Identification of *Staphylococcus.*

1st day. 1. Make a Gram stain of the infected material supplied. Note whether the bacteria are abundant and whether they are situated intracellularly or extracellularly. If the specimen examined is urine, peritoneal fluid, etc., determine microscopically what types of cells are present.

2. Plate the specimen on a plain agar and on a blood agar plate. Use the plain agar plate as the original plate and the blood plate as the "1st" dilution plate. Incubate 24 hours.

2d day. 1. Examine plates inoculated yesterday. Note the size, form, and color of the colonies. Is the blood hemolyzed? What type of hemolysis is produced?

2. Examine the colonies which have developed, by Gram's method. Note the arrangement of the organisms in clusters. Do they stain uniformly?

3. Make transplants from a pure colony to veal infusion broth, Dunham's peptone solution, nitrate broth, brom cresol purple milk and dextrose, lactose, saccharose peptone serum medium.

4. Make transplants from the *Staphylococcus aureaus* isolated last week and compare the results with those obtained from the organism isolated from the clinical material.

5. Study a pure culture of *Micrococcus tretragenus* in hanging drop preparation and stained by Gram's method. How does the arrangement of this coccus differ from that of the *Staphylococcus?*

3d day. Record the reactions, reincubate the cultures.

4th day. After 3 days' incubation, test for the presence of indol and the formation of nitrites.

XXXVI. Isolation and Identification of Organisms belonging to the *Streptococcus* Group—Throat Flora.

2d day. Let an assistant swab your tonsils or the lymphoid tissue of the pharynx even if tonsillectomized. Sterile swabs and sterile tongue depressors will be available. Make 2 dilution spreads on the blood agar plates prepared last week. Use the so-called 'ray spreading method' so that well-isolated colonies will develop. Incubate 48 hours.

3d day. Examine the blood agar plates seeded with throat material.

1. Distinguish as many different types of colonies as possible. Describe their appearance and examine them by Gram's method.

2. Transfer a hemolytic colony to a blood agar slant and to an Avery's broth ("artificial mouse"). Incubate 18–25 hours.

3. Transfer a β hemolytic colony to a blood agar slant and to a tube of Avery's broth. If hemolytic colonies of the β type are absent on the blood agar plates, isolate one from a plate supplied by the instructor. Incubate 18–24 hours.

4th day. Make Gram stains of the cultures on the blood agar slant (separate films of the growth on the slant and that in the water of condensation) and in the Avery broth planted yesterday. Is

the isolated strain pure? What are the differences in the morphological appearance of the organisms grown on solid instead of liquid media? If the growth on the blood agar slants appears to be pure, test the action produced by each strain in milk, and in salicin, saccharose, lactose, raffinose, inulin, and mannite Hiss serum peptone broth. Incubate at 37° C. and examine cultures after 24 hours, 3 days, and 5 days. Test a portion of the growth in Avery's broth for bile solubility in the manner described on pages 156, 162. The bile solubility test is one of the most important means of differentiating certain members of the *pneumococcus* from the *streptococcus* group.

If the culture is contaminated, purify it from the Avery's broth or from the water of condensation of the blood agar slant by the dilution method. Proceed in the following manner. Melt 3 tubes of agar containing 12–15 c.c. of veal infusion agar. Cool to 45° C. Add 1 c.c. of warmed sterile blood (heated also to 45° C.). Then make dilution pour plates in the usual manner. (See p. 34.) Incubate 24 hours and proceed to isolate the purified colony as previously described. Great care must be taken to avoid extraneous contamination in carrying out this technic. Why is the dilution method of purification more reliable than the surface streak method? Is there any difference in the type of colony formed in the depths and on the surface of the plates?

During the week and for the next two or three weeks, swabs carrying excretions, exudates, or pus from patients or animals infected with staphylococci or streptococci will be assigned to students for diagnosis. The procedure followed will depend on the nature of the specimen. See Part IV.

If possible throat swabs from scarlet fever patients will be obtained and given to students for diagnosis. The method of isolation and cultural study is the same as that outlined above. The final identification depends on agglutination and toxin tests, which will be demonstrated by a group of students.

References:

Bloomfield, A. L. Definition of hemolytic Streptococcus parasitism in upper air passages of healthy people. *Arch. Int. Med.*, 1923, 32, p. 386.

Brown, J. H. The use of blood agar for the study of streptococci. Rockefeller Institute for Medical Research, *Monograph* No. 9, New York, 1919.

Dick, G. F., and Dick, G. H. Method of recognizing scarlet fever streptococci by means of specific toxin production. *Jour. Am. Med. Assoc.*, 1925, 84 p. 802.

Gay, F. P. Recent aspects of Streptococcus infection. *Jour. Lab. and Clin. Med.*, 1918, 3, p. 3.

Stevens, F. A., and Dochez, A. R. Studies on biology of Streptococcus. *Jour. Exper. Med.*, 1924, 40, p. 253 and p. 493.

Eleventh Week

XXXV. Complete the study of the *Staphylococcus* cultures.

XXXVI. A. Complete the identification of at least one alpha and one beta strain of *Streptococcus.*

B. Each student will submit to the Dick test for scarlet fever susceptibility.

XXXVII. Study of the *Diplococcus pneumoniae.*

XXXVIII. Study of *B. pneumoniae* "Friedlander."

Special Materials Required—

Sterile mortars and pestles.
Centrifuge tubes.
Sterile bile (rabbit or ox).
Blood media (No. 6) as designed in tenth week.
Hiss serum water (No. 16) carbohydrate media as designed in the tenth week.
Glucose veal broth P_H 7.8 (No. 29).
Pneumococcus antisera Types I, II, and III.
Pure cultures of pneumococcus Types I, II, III, IV, grown in Avery's broth.
Mice infected with pneumococci Types I, II, III, or IV.
Friedlander's bacillus grown in Sabouraud agar (No. 63).
Normal mice.

XXXV and XXXVI A. Complete the Identification of the Staphylococcus and Streptococcus Cultures as indicated in the preceding pages.

XXXVI. B. Dick Test for Scarlet Fever. For procedure see problem 65.

Reference:

Zingher, A. The Dick test in normal persons and in acute and convalescent cases of scarlet fever. Immunity results with scarlet fever toxin. *Jour. Am. Med. Assoc.*, 1924, 83, p. 432.

XXXVII. Study of *Diplococcus pneumoniae.*

1st day. A. The cultural and serological characteristics of pure cultures of *Diplococcus pneumoniae* are studied on pure cultures in Avery's broth. Proceed as follows:

1. Make Gram stains; note shape and arrangement of cocci.
2. Make capsule stains.

3. Transplant 3 loopfuls of the culture to a blood plate, dalli over the surface of the first plate, then to second plate. Incubate for from 18 to 24 hours at 37° C.

4. Transplant 0.1 c.c. of the 18-hour-old culture to a fresh "artificial mouse."

NOTE: Cultures of pneumococcus in Avery's broth may die off rather rapidly. To perpetuate cultures in this medium it is therefore advisable to use a heavy inoculum (about 0.1 c.c. of the liquid culture) for 5 c.c. of fresh medium in making transplants.

5. Determine the type of pneumococcus present by the methods outlined below.

(*a*) Macroscopic agglutination test and bile solubility.

(*aa*) Centrifugalize the Avery broth culture at low speed for about 3 minutes to throw down the débris and the blood cells.

(*bb*) With a Pasteur pipette and safety cap transfer the supernatant fluid containing the bacteria to a sterile centrifuge tube.

(*cc*) Centrifugalize at high speed for 30 minutes.

(*dd*) With a Pasteur pipette remove the supernatant fluid and save for the precipitin test. Emulsify the sedimented bacteria in salt solution making up the volume approximately to that of the original culture (at least 4 c.c. are necessary for the test).

(*ee*) With the resuspended organisms carry out the macroscopic agglutination test in the following manner:

To each of a series of small test tubes add 0.5 c.c. of the serums, bile, etc., respectively as indicated in the table.* Then add 0.5 c.c. of the bacterial suspension. Incubate at 56° C. for 2 hours. The bile tube must be examined after 30 to 60 minutes' incubation. Read the reactions and note the results.

---

* To obviate the difficulty which occasionally arises from the occurrence of Group IV strains that show cross-agglutination in all three types of immune serum, the optimum dilutions of serum that will surely identify all type strains but fail to give any cross-agglutination reactions have been determined on a large series of strains. (This work applies only to the antipneumococcus immune serum prepared by New York State Department of Health.)

The results with Subgroup II pneumococci have not been so satisfactory. Reference to the table on page 162 will show that pneumococci belonging to these groups give a precipitin reaction with undiluted Type II serum but not in the 1 to 10 dilution, thereby being distinguishable from Type II pneumococci. A number of Subgroup II organisms, however, have been encountered in which the peritoneal washings have failed to give a precipitin reaction with undiluted Type II serum. In the identification of the fixed parasitic types of pneumococci this occasional difficulty is of little practical importance from the point of view of treatment, as there is at present no specific therapy for cases of pneumonia caused by pneumococci of the subgroups II. For purposes of classification and statistics these organisms can readily be identified subsequently when the organism has been obtained in pure culture.

TABLE 9

DETERMINATION OF PNEUMOCOCCUS TYPES BY THE AGGLUTINATION TEST

	Serum Type I (1 to 10) 0.5 c.c.	Serum Type II (undiluted) 0.5 c.c.	Serum Type II (1 to 10) 0.5 c.c.	Serum Type III (1 to 5) 0.5 c.c.	Normal horse serum (1 to 5) 0.5 c.c.	Sterile ox bile 0.1 c.c. saline 0.4 c.c.	Sterile saline 0.5 c.c.
	+	+	+	+	+	+	+
	0.5 c.c. bacterial suspension added to each tube.						
Type I	++	−	−	−	−	sol.	−
Type II	−	++	++	−	−	sol	−
Subgroups II A, B, X	−	+	−	−	−	sol	−
Type III	−	−	−	++	−	sol	−
Group IV	−	−	−	−	−	sol	−

(*b*) Precipitin test carried out with the clear supernatant fluid (from step (*dd*) in (*a*)).

(*aa*) Into a series of small agglutination tubes add the following dilution of antipneumococcus serum.†

Tube 1—0.5 c.c. Type I diluted 1:10.

Tube 2—0.5 c.c. Type II undiluted.

Tube 3—0.5 c.c. Type II diluted 1:10.

Tube 4—0.5 c.c. Type III diluted 1:5.

Tube 5—0.5 c.c. normal horse serum diluted 1:5.

(*bb*) Layer 0.5 c.c. of the clear supernatant fluid from the Avery broth culture on to each of the latter sera. If the type be either I, II, or III, a white ring will form at the point of contact of the serum and the supernatant fluid. Type IV does not react. Incubation is usually not necessary. If the reactions do not occur immediately the tubes may be placed in a water bath at 37° C. for 20 minutes and then read. A positive precipitin test in tube 2 and not in tube 3 indicates Subgroup II.

2d day. 1. Examine the blood plates inoculated yesterday from the Avery broth. Note the type of colony. Study its structure and the type of hemolysis under low power. Stain fixed preparations of the growth by Gram's and by Hiss's capsule stain. Are capsules present? Under what conditions are capsules formed? What is the value of the demonstration of a capsule in this group?

† The exact dilution will vary according to the batch of serum.

2. If the growth is pure make transplants to the media used to differentiate the members of the Streptococcus group. If a contaminating colony has grown on the blood agar plate, pick a well isolated pneumococcus colony to a blood agar slant. Incubate 24 hours.

3d day. Examine the differential media inoculated with the pneumococcus culture. Note reactions, reincubate and read again after 3 and 5 days' growth.

B. Isolation and Identification of *Diplococcus pneumoniae* by mouse inoculation.

1st day. Mice previously injected intraperitoneally by the instructor will be available.

1. If the animal is not dead but sick aspirate a small amount of peritoneal fluid with a Pasteur pipette, make a Gram stain and determine whether the growth of pneumococci in the peritoneal fluid is heavy enough to furnish sufficient organisms to carry out the agglutination test. If there are sufficient organisms present chloroform the mouse and proceed as in step (2).

2. If the mouse is dead perform an autopsy and determine the type of pneumococcus responsible for the infection in the following manner:

(*a*) *Mouse autopsy.*—Prepare the animal in the usual manner, dissect the skin from the abdominal wall, and open the thoracic cavity. Cultures are made from the heart's blood in glucose veal broth, P_H 7.8 and on a blood agar plate. The peritoneal cavity is opened with sterile precautions, and cultures are made of the exudate in Avery's broth and on a blood agar plate. Smears are made and stained by Gram's method and by Hiss's capsule stain. The peritoneal exudate is then washed out with 4 or 5 c.c. of sterile salt solution by means of a sterile Pasteur pipette, the washings are placed in a centrifuge tube.

(*b*) *Determination of type.*—

(*aa*) *Agglutination technic.* When the pneumococcus is present in pure culture in the peritoneal exudate the determination of the type may be satisfactorily made by the macroscopic agglutination tests as follows: The peritoneal washings are centrifugalized at low speed for a few minutes until the cells and fibrin contained in the exudate are thrown down. The supernatant bacterial suspension is decanted into a

second centrifuge tube and centrifugalized at high speed until the organisms are thrown down. The supernatant fluid is decanted into another tube and the bacterial sediment taken up in sufficient sterile salt solution to make a moderately heavy suspension. The concentration of bacteria should be similar to that of a good eighteen-hour broth culture of the pneumococcus. This suspension is used directly for macroscopic agglutination tests, being mixed with immune serum in small test tubes in equal quantities of 0.5 c.c. each in the manner described on page 157.

(*bb*) *Precipitin technic.* If the mouse has been dead for some time and if the peritoneal fluid upon microscopic examination does not prove to be satisfactory for use as an antigen in the agglutination test proceed in the following manner:

1. Take heart blood cultures in glucose veal broth and on a blood agar plate.

2. Culture a loopful of the peritoneal exudate in an Avery broth tube and on a blood agar plate. (Shake dilution method.)

3. Culture a bit of the spleen on a blood agar plate. The remainder of the spleen may be carefully removed, placed in a sterile test tube and saved for future use. It has been found that the pneumococcus will remain viable in the dried spleens for many months.

4. Wash out the peritoneal cavity with about 3 c.c. of sterile saline solution, centrifugalize at high speed and use the clear supernatant fluid for the precipitin test. See page 157.

2d day. Proceed with the isolation of a pure culture from the blood agar plates inoculated yesterday in the manner described on page 157 (procedure for second and third days) and test the action of the purified strain on the differential mediums. See page 150.

C. The Isolation of Pneumococcus from Sputum Cultures

Students who attempt to demonstrate pneumococcus in sputums will study pure cultures of pneumococcus instead of mice infected with this organism.

1st day. A group of students under the guidance of a leader will pool their sputums and inoculate a mouse intraperitoneally. Proceed in the following manner:

(*a*) *Collection of sputum from adults.*—Care should be exercised in the collection of sputum to obtain a specimen from the deeper air passages as free as possible from saliva. This can be done in practically all cases, even the most difficult, with a little persistence. The mouth is first rinsed with sterile water. The sputum is collected in a sterile Petri dish or other suitable container and should be injected at once. When delay is unavoidable, the specimen should be kept on ice during the interval.

(*b*) *Collection of sputum from children.*—The mouth is rinsed first with sterile water or with a weak solution of sodium bicarbonate. The sputum which gathers in the upper region of the larynx is collected by means of a sterile swab.

(*c*) *Microscopic examination of the sputum.*—Direct smears are made from the sputum and stained by Gram's method, using 10 per cent aqueous safranin as a counter-stain, by Ziehl-Neelsen's and by Hiss's capsule stain. This serves to give an idea of the nature of the organism present and an indication of the source of the sputum. Suitable lung specimens are relatively free in most instances from contaminating mouth organisms. It is frequently possible to identify Type III (*Pneumococcus mucosus*) organisms when they are present, as they possess a very large distinct capsule stained by both Gram's and Hiss's method.

(*d*) 1. First Method of demonstrating *Diplococcus pneumoniae* in Sputum.

Mouse inoculation.—A small portion of the sputum, about the size of a bean, is selected and washed through three or four changes of sterile salt solution in sterile Petri dishes, to remove the surface contaminations. The washed sputum is then tranferred to a sterile mortar, ground up and emulsified with about 1 c.c. of sterile bouillon or salt solution, added drop by drop, until a homogeneous emulsion which will readily pass through the needle of a small syringe is obtained. From 0.5 to 1 c.c. of this emulsion is inoculated intraperitoneally into a white mouse with a sterile syringe. The pneumococcus grows rapidly in the mouse peritoneum while the majority of saprophytic mouth organisms rapidly die off with the exceptions noted: *B. influenzae* and occasionally *M. catarrhalis, Staphylococcus,* and *Streptococcus.*

Pneumococcal invasion of the blood stream also occurs early. *B. influenzae* likewise invades the blood stream if present; other organisms, as a rule, do not. The time elapsing before there is a sufficient growth of the pneumococcus in the mouse peritoneum for the satisfactory determination of type varies with the individual case, depending upon the abundance of pneumococci in the specimen

of sputum and the virulence of the strain present. This may be anywhere from five to twenty-four hours, averaging six to eight hours, with the parasitic fixed Types I, II, and III. As soon as the injected mouse appears sick a drop of peritoneal exudate is removed by means of peritoneal puncture with a sterile capillary pipette, spread on a slide, stained by Gram's method, and examined microscopically to determine whether there is abundant growth of pneumococcus; the mouse is killed and the determination of the type proceeded with. If the growth is only moderate or if other organisms are present in any quantity, further time must be allowed until subsequent examination of the peritoneal exudate shows an abundant growth of pneumococcus. It should be emphasized that undue haste in killing the mouse is time lost in the end. Perform an autopsy. Proceed according to the method outlined on page 158.

2d, 3d, or 4th days. (2) Second Method of Demonstrating *Diplococcus pneumoniae* in sputum.

*Determination of type of pneumococcus by Avery's rapid cultural or "artificial mouse" method.**

(*a*) Collection of sputum same as for C (*a*). Take care to obtain a specimen from the deep air passages and to avoid mouth secretions.

(*b*) A portion of the sputum the size of a bean should be selected and washed even more carefully than for the mouse inoculation, passing it 3 or 4 times through sterile salt solution, or preferably through tubes of sterile broth.

(*c*) The sputum is then ground in a sterile mortar, adding 0.5–1.0 c.c. of broth drop by drop. This emulsion is then aspirated with a 1 c.c. or with a Pasteur pipette and introduced directly into warmed rabbit's blood glucose broth medium (Avery broth). One drop is plated on blood agar plate (2 dilution streak plates).

(*d*) Incubate the Avery broth (also called "artificial mouse") in a water bath kept at 37.5° C. for from 5 to 6 hours. Make smears of the growth, stain with Gram's stain, and prepare a blood agar plate (streak dilution method). Incubate blood plate at 37.5° C. for 18–24 hours. Examine according to the method outlined on page 157. How do you explain the difference in the types which develop before and after enrichment of the cultures?

* AVERY, O. T. Types of pneumococcus. *Jour. Am. Med. Assoc.*, 1918, 70, p. 17.

(*e*) Determination of the type of pneumococcus by the precipitin test.

(*aa*) Centrifugalize the 5-hour-old culture at low speed for 2 minutes—just enough to throw down the red blood cells but not enough to bring down the bacteria.

(*bb*) Add 2–3 c.c. of the supernatant fluid to 1 c.c. of sterile fresh bile, diluted 1:5, and place the mixture in a water bath at 37° C. for 20 minutes. If not clear at this time centrifuge at high speed. The clear supernatant is then used for a precipitin test according to the method described on page 157.

(*f*) Determination of the type of pneumococcus by the agglutination test.

An agglutination test as described on page 156 may be made directly on the 5-hour-old sputum culture after removal of the red blood cells.

C. Determination of Types of Pneumococcus by Means of Specific Precipitin Reaction in the Urine

A certain percentage of patients suffering from lobar pneumonia excrete in their urine at some stage of the disease a soluble substance of pneumococcus origin. This substance gives a specific precipitin reaction with antipneumococcic serum corresponding in type to the organism with which the individual is infected. The soluble substances may appear in the urine at as early a period as 12 hours after the initial chill, or it may appear for the first time at a later stage of the disease. It may continue to be excreted for many days after recovery has occurred. It is the rule to find this substance in the urine when pneumococcus septicemia exists. Examination of the urine for the presence of this substance has proved of some value for the rapid determination of the type of pneumococcus with which the individual is infected. This test, however, should not supplant the usual bacteriologic methods for the differential diagnosis of pneumococcus types, but is useful in that in a certain percentage of instances an immediate diagnosis can be made from an examination of the urine.

TABLE 10

DETERMINATION OF PNEUMOCOCCUS TYPES BY PRECIPITIN REACTION IN URINE

0.5 c.c. urine from:	Serum I 0.5 c.c.	Serum II 0.5 c.c.	Serum III 0.5 c.c.
Type I pneumonia	+	−	−
Type II pneumonia	−	+	−
Type III pneumonia	−	−	+
Type IV pneumonia	−	−	−

Method using unconcentrated urine.—A specimen of urine is obtained as soon as possible and cleared by centrifuging. The clear urine is mixed in quantities of 0.5 c.c. each with equal amounts of antipneumococcic serum of the different types in a series of agglutination tubes.

The strength of the reaction may vary from an almost imperceptible cloud to a heavy flocculent precipitate. The reaction may occur in some instances immediately on the mixing of urine and serum, or may require incubation in the water bath at 37° C. for 1 hour. Prolonged incubation, however, must be avoided, since bacterial growth under such conditions may obscure the test. In the precipitin reaction it is essential that all the reagents used, including the immune sera, should be water-clear. In case the reaction is negative or so faint as to be indecisive with the whole urine, the following method of concentrating the urine may be employed:

Concentration of urine for precipitin test.—25 c.c. or more of a 24-hour specimen of urine, with the addition of a few drops of acetic acid, are boiled down to a volume of about 5 c.c., filtered through paper to remove any precipitate of albumen that may occur, and the filtrate is added to 8 to 10 volumes of 95 per cent alcohol. The precipitate which forms is collected by centrifuging, rapidly dried to remove the excess of alcohol, and the residue extracted with 2 to 3 c.c. of salt solution, which redissolves the specific substance. Any undissolved material is removed by centrifuging and the clear salt solution extract is used for the precipitin test, as shown in the table.

References:

Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R. *Acute Lobar Pneumonia.* Rockefeller Institute for Med. Research, *Monograph* No. 7, 1918.

Glynn, E. E., and Digby, L. *Bacteriological and Clinical Observations on Pneumonia and Empyemata, with special reference to the Pneumococcus and to Serum Treatment.* Medical Research Council Special Report Series No. 79, London, 1923.

XXXVIII. Study of a Pure Culture of *B. pneumoniae* ("Friedlander.")

3d day. A pure culture of Friedlander's bacillus grown on Sauboraud agar will be supplied.

1. Make smears and stain by Gram's method.
2. By Hiss's capsule stain.
3. Study its action on the media usually employed for the identification of *B. coli.*

Reference:

Small, J. C., and Julianelle, L. A. Biologic and serologic studies of Bacillus mucosus group. *Jour. Infect. Dis.*, 1923, 32, p. 456.

Twelfth Week

XXXVIII. Complete the study dealing with the identification of *Diplococcus pneumoniae.*

XXXIX. Study of a pure culture of *Micrococcus intracellularis meningitidis.*

XL. Methods of detecting Meningococcus carriers.

XLI. The identification of microorganisms in specimens of spinal fluid.

XLII. Study of hemoglobinophilic bacteria.

A. *B. influenzae.*

B. *B. pertussis.*

XLIII. Methods of isolating *B. influenzae.*

Special Materials Required—

West tubes.
Blood agar plates and slants (No. 6).
Cooked blood agar plates and slants (No. 7).
Laked blood agar plates (No. 8).
Sodium oleate blood agar plates (No. 67).
Glucose, Levulose, Maltose, Saccharose } Hiss serum water + 1 per cent Andrade's indicator (No. 16).
Veal infusion agar (No. 76).
Veal infusion broth (No. 77).
Cooked blood veal infusion broth (No. 9).
Polyvalent meningococcus serum.
Normal horse serum.

XXXVIII. Complete the Identification of the Pneumococcus Types in Sputum Cultures.

XXXIX. Study of a Pure Culture of *Micrococcus intracellularis meningitidis.*

1st day. 1. Laked blood agar plates will be supplied. Seed lightly with the pure cultures of meningococci supplied. Incubate 24 hours at 37° C.

TABLE 11

Fermentation Reactions* of the More Important Gram Negative Diplococci

	Name of organism†	Dextrose	Maltose	Levulose	Saccharose	Remarks
	Gonococcus [Neisseria gonorrheae]	+	−	−	−	Sometimes very difficult to cultivate. Grows only on special media such as blood, starch, testicular agar, etc., and grows best in a slightly increased CO_2 tension.
	Micrococcus intracellularis meningitides [Neisseria intracellularis]	+	+	−	−	Grows fairly well on blood or serum agar. Little difficulty is encountered if the specimens and media are constantly kept at body temperature.
	Micrococcus catarrhalis. [Neisseria catarrhalis]	−	−	−	−	
	Micrococcus pharyngis siccus [Neisseria sicca]	+	+	+	+	Colonies gray, and dry, tend to adhere to the media.
Micrococcus Flavus	Chromogenic Group I [Neisseria perflava]	+	+	+	+	On dextrose agar colonies greenish gray by reflected light, greenish yellow and opaque by transmitted light.
Micrococcus Flavus	Chromogenic Group II [Neisseria flava]	+	+	+	−	On dextrose agar colonies greenish gray by reflected light, greenish yellow and opaque by transmitted light.
Micrococcus Flavus	Chromogenic Group III [Neisseria subflava]	+	+	−	−	On dextrose agar colonies greenish gray by reflected light, greenish yellow and opaque by transmitted light.

* The organisms should be grown in Hiss serum water media containing 1 per cent Andrade's indicator and 1 per cent of the test substance. + indicates the production of acid, − indicates failure to ferment the test substance. Gas is not produced by members of this group.

† The more common name is given first. The name given in brackets [ ] is the one proposed by the Society of American Bacteriologists—Bergey's *Manual*.

2d day. 1. Examination of plate cultures. Study the isolated colonies that develop on the plate seeded yesterday. The colonies of the meningococcus are small and delicate. They are convex, glistening, translucent, and as they grow older tend to become opaque in the center. The young colonies may be confused with those of *Diplococcus crassus, Micrococcus catarrhalis, Diplococcus pharyngis flavus, Diplococcus pharyngis siccus,* and some streptococci.

2. Stain suspicious colonies by Gram's method.

3. Transplant a colony to a blood slant (warm the slant before using it as meningococci are very sensitive to low temperature). Incubate 24–48 hours. If other Gram negative diplococci are encountered make transplants to blood agar slants and identify by the reaction in differential mediums used to identify Gram negative cocci.

4. Conduct presumptive agglutination test on slides with polyvalent meningococci serum.

Presumptive slide agglutination test.—Drop A is 1/10 dilution of of polyvalent meningococcus serum. Drop B is a 1/10 dilution of normal horse serum (control). Drop C is 0.85 per cent salt solution (control).

Three drops are placed on a microscopic slide as shown. The suspected colony is fished and the platinum wire is dipped into each drop to permit part of the colony to wash off into it. In doing this, dip first into drop C, then into drop B, and finally into drop A. Burn the wire, and after it has cooled, emulsify the material in each drop thoroughly, working in the same direction. Agglutination in A and none in B and C is presumptive for meningococcus. Any other reactions are negative and indicate another type of organism. The slide can be stained by Gram's method to verify the morphology and staining characteristics of the organism present.

Macroscopic agglutination.—To 1 c.c. quantities of an emulsion of meningococci in five tubes add:

1. To the first tube, 1 c.c. of a 1/100 dilution of normal type meningococcus serum.

2. To the second tube, 1 c.c. of a 1/100 dilution parameningococcus serum.

3. To the third tube, 1 c.c. of a 1/50 dilution of Rockefeller polyvalent antimeningococcic serum.

4. To the fourth tube, 1 c.c. of a 1/10 dilution of normal horse serum (control).

5. To the fifth tube, 1 c.c. salt solution (control).

Incubate at 37° C. for 3 hours.

Interpretation.—Agglutination in tubes 1 and 3 = meningococcus (normal).

Agglutination in tubes 2 and 3 = parameningococcus.

Agglutination in tube 3 = meningococcus (intermediary).

Agglutination in all tubes, in none, or in either of the two controls is negative for meningococcus.

3d day. Examine blood agar slants inoculated last period and if pure identify the species by means of the following mediums: glucose, maltose, levulose, saccharose serum medium, plain broth, and plain agar.

XL. Methods of Detecting Meningococcus Carriers. Exercise to be conducted by one-half of the class.

MENINGOCOCCUS ISOLATION AND CARRIER DETECTION

1st day. Obtain material on a swab from the posterior nasopharynx (adenoid region); plate directly from the swab, and after 24 hours' incubation of the inverted plate, make presumptive slide agglutination tests with specific sera and suitable controls, using colonies fished directly from the plates. The dried drops are later fixed and stained to determine whether the organism is a Gram negative diplococcus. Where time and material are available, this technic is elaborated.

Swabbing.—In skilled hands the 'open' swab may be successfully employed, but the use of the West tube is recommended. The West tube is prepared in the following manner: The ends of a glass tube (about 15 cm. long, 8 mm. inside diameter) are rounded in the flame and bent at an angle of about 135°, about 5–6 cm. from one end (this is the swab end of the tube). A cotton swab is fixed on the end of a copper wire cut a little longer than the glass tube. This is inserted in the tube; both ends are plugged with cotton; the tube is wrapper in paper and sterilized for 30 minutes at 15 pounds pressure. The material should be collected from the posterior naso-pharynx behind the soft palate, and swab must not touch lips, tongue, tonsils, etc. The mucous material on the tip of the swab is immediately

inoculated by touching the surface of the specially warmed medium in a Petri dish. The material is then spread over the surface with a platinum wire or it may be spread directly with the swab. The plates should be immediately incubated in the inverted position. Cooling of the plates and delay in incubation diminishes the number of successful isolations of the meningococcus.

References:

EASTWOOD, A. Second report on bacteriological aspects of the Meningococcus carrier problem. *Jour. Hyg.*, 1918, 17, p. 63.

GORDON, M. H. Identification of the Meningococcus. *Jour. Hyg.*, 1918, 17, p. 290.

TULLOCH, W. J. A study of the mechanism of the agglutination reaction together with an examination of the efficicacy of these tests for identifying specimens of the meningococcus isolated from 354 cases of cerebro-spinal fever. *Jour. Hyg.*, 1918, 17, p. 317.

FLEXNER, S. Mode of infection, means of prevention and specific treatment of epidemic meningitis. *Jour. Am. Med. Assoc.*, 1917, 69, pp. 639, 721, 817.

WEST, C. E. *Proc.* Roy. Soc. Med., Otological Section, 1910–11, 4, p. 43.

XLI. The Identification of the Microorganisms in Specimens of Spinal Fluid.

1st day. Students will be requested to study specimens of spinal fluids secured from clinical cases of meningitis.

The procedure of examinations is as follows:

1. Examine the fluid for physical changes as color, foam, pellicle formation on standing.

The fluid is either permitted to stand and the pellicle or delicate clot is used for examination, or it is incubated for several hours and then examined. In the majority of cases a sample of the spinal fluid is placed in sterile centrifuge tubes and immediately centrifugalized. From the sediment smears are made and one of these is stained by Gram's method, one is fixed in alcohol-ether and stained with a polychromatic stain (Wright's or Giemsa). The third is stained by Konrich's and the fourth by Hiss's method (capsule stain). According to the findings, blood agar slants or other media are used.

The cerebrospinal fluid in various diseases.—

(*a*) *Tuberculous meningitis.* Fluid clear and transparent; heavy foam on shaking. On standing definite clot or pellicle formation. Tubercle bacilli are more readily found in the pellicle than in the sediment.

(*b*) *Meningococcus meningitis.* Fluid clear to slight opalescence; dome-shaped, reticulated pellicle in the early stages. Later in the

course turbid, yellowish green; on standing pronounced firm and spreading sediment, sometimes with granules and greenish in color. Leucocytes are increased in number; Gram negative biscuit-shaped organisms within the leucocytes, some also outside of the cells. Sometimes early in the disease the organisms in the direct smear may be very scanty in number. After the first or second dose of antimeningococci serum, there is usually a shower of organisms in the fluid. Cultivation on serum or blood agar slants is not always easy. Enrichment in the diluted cerebrospinal fluid is recommended before seeding the blood agar slants. Mixed infection with pneumococci have been observed.

(*c*) *Pneumococcus meningitis.* As a rule the spinal fluid is pearly gray; on standing a heavy fibrinous sediment forms which is usually larger than in the meningococcic form of meningitis. The bacteria are often arranged in chains of four or six resembling streptococci; the capsule stain may be used to differentiate the two bacteria. The final diagnosis is made by cultures and agglutination tests.

(*d*) *Streptococcus meningitis.* Fluid is very turbid; the cells are increased; blood agar cultures are used for differentiation; hemolytic are more frequent than non-hemolitic streptococci.

(*e*) *Influenza meningitis.* Fluid opalescent to distinctly yellow, cells mostly polymorphonuclear leucocytes; Gram negative bacilli are found extracellularly in the direct smear; a special type of hemoglobinophilic organisms grows on hemoglobin containing media.

(*f*) *Colon-typhoid meningitis.* Fluid similar to other types of meningitis. The diagnosis is established by the cultivation and specific agglutination of the bacteria.

Reference:

LEVINSON, A. *Cerebrospinal Fluid in Health and Disease.* Ed. 2; St. Louis, G. V. Mosby Co., 1922, p. 150, etc.

XLII. Study of Hemoglobinophilic Bacteria.

1st day. A. Each student will study pure and mixed cultures of *B. influenzae.*

1. Make Gram stain of the pure culture supplied.

2. Transplant a loopful of the culture to a cooked blood agar plate. Make a dilution plate to sodium oleate agar. Incubate 24 hours at 37.5° C.

2d day. 1. Examine well-isolated colonies macroscopically and make Gram stains of the growth.

2. Transplant to a cooked blood agar slant and incubate for 24–48 hours at 37° C.

3d day. Make a Gram stain of the growth on the cooked blood agar slant. If pure, transplant to cooked blood broth, plain agar and plain broth. Note the differences in growth. Incubate another 3 days and then test the cooked blood broth for the presence of indol. What prerequisites are necessary for the cultivation of *B. influenzae?* What is meant by the satellite phenomena? Explain the reaction.

XLII. B. Make Gram stain of a pure culture of *B. pertussis.* How could you differentiate *B. pertussis* from *B. influenzae?* Outline in the day-book the media to be used for differentiation.

If available, specimens or cultures of the Morax-Axenfeld and the Ducrey bacillus will be distributed for study.

Reference:

KRISTENSEN, M. *Investigations into the Occurrence and Classification of the Haemoglobinophilic Bacteria.* Levin and Munksgaard, Copenhagen, 1922.

2d day. Study growth on the plates in the manner described under XXXIX.

XLIII. Methods of isolating *B. influenzae.*

Exercise to be conducted by students who are not engaged on Exercise XL.

1st day. Prepare cooked blood agar plates. Sodium oleate blood agar plates will be supplied. Make 2 dilutions on a cooked blood agar plate and a sodium oleate blood agar plate with throat swabs of students who show small Gram negative rods in direct microscopic smears. Incubate 24 hours at 37.5° C.

2d day. Study the different types of colonies that develop and make smears. Stain by Gram's method. If small round slightly elevated transparent or slightly grayish colonies, composed of rather slender Gram negative rods, are present make transplants to cooked blood agar slants and incubate 24–48 hours. Do not attempt to keep the culture for a week without frequent transplanting as the strains often die out in from 3 to 4 days.

3d day. Make Gram stain. If pure transplant to cooked blood broth medium, plain agar, and plain broth. Incubate 24 hours.

4th day. Note the difference in growth. Incubate the cooked blood broth for another three days. Then test for the presence of indol.

Thirteenth Week

XXXIX. Study of cultural reactions of meningococci, etc. (completed).

XLIV. Study of *Diplococcus gonorrhoeae.*

XLV. Study of pathogenic branched bacteria.

XLVI. Study of *B. mallei.*

XLVII. Study of pathogenic fungi.

Special Materials Required—

Glass chambers for the preparation of hanging blocks.
Sabouraud's agar (No. 63).
Pure culture of gonococcus.
Pure cultures of Streptothrix.
Sporothrix beurmani.
Pure cultures of:
- *Torula hominis.*
- *Monilia psilosa.*
- *Monilia albicans.*
- *Blastomyces hominis.*
- *Coccidioides immitis.*

Vaginal smears.
Smears from acute and chronic cases of gonorrhoea.
Male guinea pig infected with *B. mallei* and cultures of *B. mallei* on potatoes.
Guinea pigs infected with *Torula hominis.*
Guinea pigs infected with *Coccidoides immitis.*
Guinea pigs infected with *Sporothrix beurmani.*
Lacto-phenol.

XXXIX–XLIII. Study of Cultural Reaction, etc. (Completed).

1st day. Complete the study dealing with the cultural reactions of meningococci, Gram negative cocci isolated from the nasopharynx and *B. influenzae* (indol test in blood broth).

XLIV. Study of *Diplococcus gonorrhoeae.*

2d day. 1. Smears of gonococcic pus will be supplied. Stain with methylene blue, thionin, and Gram stain, counterstain with neutral red.

2. Vaginal smears will be available. Stain with methylene blue, thionin, and Gram stand. Note the types of organisms present, their staining reactions, and the relation of the organisms to the leucocytes. What means could one employ in differentiating between the gonococcus and meningococcus?

Properly qualified students will have an opportunity to cultivate the *Diplococcus gonorrhoeae* by the methods of Torrey, *Jour. Infect. Dis.*, 1922, 31, p. 125.

Reference:

The Laboratory Diagnosis of Gonococcal Infections. Medical Research Committee, Special Report No. 19, new ed., 1923.

XLV. Study of Pathogenic Branched Bacteria. Specimens from "lumpy jaw," and a culture of *Streptothrix madurae* or Eppinger will be supplied.

Scrape the tartar from teeth of individuals with inflamed gums (*Leptothrix* and *Cladothrix*). Make thin films and stain by Gram's method and with dilute methylene blue.

Reference:

Ørskov, J. *Investigations into the Morphology of the Ray Fungi.* Levin and Munksgaard, Copenhagen, 1923.

XLVI. Study of *B. mallei.*

The instructor will autopsy a male guinea pig which has previously been inoculated with *B. mallei.* Smears will be prepared and furnished to the students after they have been fixed in bichloride solution. The slides must be carefully washed in running water before staining with methylene blue and by Gram's method. Cultures on potato slabs and on glycerine agar sterilized with formalin will be available for a comparative study.

XLVII. Study of Pathogenic Fungi.

A. Morphology and Staining Reactions.

1st day. Note the type of growth of the cultures supplied and make

1. Hanging drop preparations in salt solutions.
2. Hanging drop preparations in dilute neutral red.
3. Hanging drop preparations in lacto-phenol solution.
4. Smears stained with simple stains, by Gram's method, acid fast stain of the following types of fungi:

(*a*) *Torula hominis.*
(*b*) *Monilia psilosa.*
(*c*) *Monilia albicans.*
(*d*) *Blastomyces hominis.*
(*e*) *Sporothrix beurmani.*

B. Cultivation of Fungi in Hanging Block Preparation. (Study *Sporothrix, Monilia albicans, Monilia psilosa.*)

1st day. 1. Melt a tube of Sabouraud's agar, pour a thin layer (2–3 mm.) aseptically into a Petri dish, and allow it to harden.

2. Flame a cover slip carefully and allow it to cool (clean cover slips may be sterilized in the hot air oven for one hour at 170° C.) This procedure is the best to insure sterility.

3. Cut out a small piece of agar about 3–4 mm. square. Place it in a sterile Petri dish and inoculate the surface of the agar with a minute amount of a suspension of the organism to be studied. The agar is then placed on the sterile cover slip in such a way that the inoculated surface touches the cover slip. After having moistened the floor of the glass chamber with a few drops of sterile water or saline, fasten the cover slip, block downward, on the vaselined rim of a Hansen chamber or an ordinary hollow ground slide. Incubate at room temperature and record the growth by drawings at frequent intervals. Note the mode of reproduction (whether by budding, endogenous sporulation, etc.) and the formation of mycelial threads.

Study at least 2 species by this method.

Special Materials Required—

Cover slip.
Vaseline.
Glass chamber.
Hanging block of agar.
Sterile water to keep chamber moist.

C. Examination of Stained Tissue Sections showing *Torula, Coccidioides immitis,* and *Actinomyces.*

3d day. Examine the tissue sections, draw the parasites. What distinguishes the growth of *Blastomyces, Coccidioides immitis,* and *Torula* in vivo?

D. Autopsy of Animals Infected with *Torula, Coccidioides immunitis, Sporothrix.*

The lesions produced by *Torula, Coccidioides immunitis,* and *Sporothrix* in guinea pigs will be demonstrated. Hanging drop preparations in 10 per cent KOH and fixed preparations of the pus should be made and stained with simple stains, by Gram's method, and with acid fast stain. Which is the best method of demonstrating these organisms in pus, etc.? Draw and describe each type studied.

Reference:

Stoddard, J. L., and Cutler, E. C. *Torula Infection in Man.* Rockefeller Institute for Medical Research, *Monograph* No. 6, 1916.

Fourteenth Week

XLVII. Complete the study of pathogenic fungi.

XLVIII. Study of anaerobic sporulating bacteria.

A. Examination of pure cultures of anaerobes.

B. Demonstration of infections produced by anaerobes in mice and guinea pigs.

C. Isolation and identification of sporulating anaerobes from garden soil.

Special Materials Required—

Burri tubes (glass tube with small rubber stoppers may be used).
Several Pasteur pipettes.
Beef heart medium (No. 5).
Brain peptone medium (No. 11).
Gelatin (No. 22).
Liver agar "deeps" (No. 46).
Milk with vaseline or a layer of cream.
Glucose, Inulin, Lactose, Mannite, Maltose, Saccharose, Salicin — Semi-solid agar containing Andrade's indicator (No. 66).
Solidified egg in peptone broth.
Pure cultures of *Cl. botulinum, sporogenes, histolyticus, oedematiens, tetanus, welchii, tertius, vibrion septique.*
Guinea pigs infected with *Cl. histolyticus, oedematiens, vibrion septique,* and *welchii.*
Mice infected with *Cl. tetani.*
Normal guinea pigs and mice.

XLVII. Complete the Study of Pathogenic Fungi as outlined in the Preceding Section.

XLVIII. Study of Anaerobic Bacteria. (For the cultivation of anaerobes see p. 38.)

A. Examination of pure Cultures of Anaerobes.

1st day. Pure cultures grown in beef heart broth, plain broth, or brain medium will be available. Each student must study at least one representative of groups A and B.

Group A	Group B
Vibrion septique	Cl. tertius
Cl. oedematiens	Cl. tetani
Cl. welchii	Cl. botulinus
	Cl. sporogenes
	Cl. histolyticus

Make hanging drop preparations of the liquid cultures and stain smears with (*a*) methylene blue or thionin and (*b*) Gram's stain. Note the appearance and odor of the cultures, the differences in staining reaction, position of the spore, etc. Liver infusion agar shake cultures of some of the types will be available for study. Note the presence or absence of gas, the size of the colonies, and with the aid of a hand lens their shape and structure.

Prepare subcultures of the following species in milk, beef heart broth, and brain medium. Prior to inoculation boil tubes for 10 minutes and cool. Why? Make shake cultures either in Burri tubes or in ordinary deep agar tubes.

2d day. Examine the agar shakes planted yesterday. The colonies should be few in number—not more than 25 in the last dilution and should be well isolated. Tubes heavily seeded are not suitable for examination as the characteristic features of a colony vary with the intensity of growth. Incubate for another 24 hours. The milk, beef heart, and brain mediums should be examined daily, and the turbidity, gas formation, odor, and proteolytic activities noted. Make Gram stains after 24 hours' incubation and again after another 3–4 days. To test for aerobic contamination make surface streaks on a peptic digest agar slant with 2–3 loopfuls of the 4–5 day culture. Incubate these aerobically for 3–4 days.

B. Demonstration of Infections produced by Anaerobes on Mice and Guinea Pigs.

2d day. Groups of students will infect pigeons, rabbits, guinea pigs, or mice with 1 c.c. of the pure cultures of the following species.

Cl. welchii	Rabbit intravenously; kill five minutes later and place the animal in the incubator at 37° C. for 16 hours.
	Pigeons intramuscularly; 10-hour-old culture is inoculated into the muscle of a pigeon. Note local lesions and general symptoms. Explain the results.
Vibrion septique	Guinea pig intramuscularly.
Cl. oedematiens	Guinea pig intramuscularly.
Cl. histolyticus	Guinea pig intramuscularly.
Cl. tetani	Mice or guinea pig on the back intramuscularly.

Note the symptoms by frequent examinations. The animals may succumb after 24–48 or 72 hours depending on the virulence of the strain.

3d day. If the animal is dead perform an autopsy. Make cultures of the following tissues or organs into beef heart medium (2 tubes) and deep liver agar shakes in Burri tubes (4 tubes, 2 originals and 2 dilutions of one of these): Site of inoculation, heart blood, liver, spleen. Heat one of the beef heart cultures and one of the original liver agar shakes at 80° C. for 10 minutes before incubation. Why? Incubate at 37° C. for 24–48 hours.

Make smears of exudate at site of inoculation, heart blood, liver surface, and stain by Gram's method. Make impression preparations of the liver and the lining of the peritoneum. Stain with simple stains and by Gram's method.

4th day. Examine the cultures inoculated yesterday. If the colonies have sufficiently developed, expel the agar from the tube by removing the rubber stopper or by gentle heating of the closed end of the tube (the procedure will be demonstrated). With the aid of a flamed razor blade cut the agar column into thin slices. Study the colony formation under low power and with the aid of a fine Pasteur pipette transfer one to beef heart medium. Pick several types (if they are present) into separate tubes, using a fresh pipette for each 'fishing.' Incubate the beef heart medium for 3–4 days.

If aerobic contaminations are present (as evidenced by growth at the surface of the agar) a different procedure may be followed. The agar column is removed by breaking the tube at the lower end in the following manner: With a diamond pencil or file scratch a ring at a level about 3 cm. below the top of the column of agar. Then place a drop of water on the scratched surface and touch it with a red-hot tip of drawn out glass tubing. The tube will crack along the marked line. Remove the agar into a sterile Petri dish by gently heating the closed end in a low gas flame. Cut thin slices of agar with the flamed razor blade and with the aid of a Pasteur pipette pick well-isolated colonies to beef heart medium and into another series of deep liver agar shakes in the manner described in the preceding paragraph. The subcultures should be grown for 48 hours, examined microscopically, replanted into liver shakes, repurified, and tested for aerobic contamination in the usual manner on agar slants and in broth.

C. A group of students will demonstrate the Isolation and Identification of Sporulating Anaerobes from Garden Soil.

1st day. 1. Mix about 5 grams of soil in 25 c.c. of sterile salt solution. Heat the suspension for 1 hour at 80° C.

2. Enrich about 5 c.c. of the heated suspension in beef heart medium. Incubate for 48–72 hours.

3d day. 1. Make a stained preparation.

2. Isolation of anaerobes by (*a*) animal inoculation, (*b*) by colony isolation.

(*a*) Animal Inoculation.

Inoculate about 1 c.c. of the enriched culture into the muscle on the hind leg of a guinea pig. See page 59. Observe the animal daily. When the animal dies, perform an autopsy and try to isolate the anaerobe by the methods described under B.

(*b*) Colony Isolation.

If spores are present in the stained preparation heat about 1 c.c. of the enriched culture at 80° C. for 15 and another for 60 minutes. Make dilution shake cultures (at least 3 dilutions—use 1–2 loopfuls as the inoculum, according to the number of spores present). Incubate 24–48 hours.

4th day. Pick well-isolated anaerobic colonies. Incubate for 48 hours or until spores have formed. If aerobic contaminations are present, the glass tube must be broken at the point below the line of aerobic growth, the lower column of agar gently heated and expelled from the tube, sections cut, and colonies picked. Prove that the organism isolated is an anaerobe and not a facultative aerobe.

Fifteenth Week

XLVIII. Continuation of the study of anaerobes.

XLIX. Examination of smears containing fusiform bacilli.

L. Study of Spirochaetes.

LI. Study of filterable viruses.

LII. Study of Rickettsia bodies.

Special Materials Required—

Fresh oysters and clams.
Smears containing fusiform bacilli.
Rabbit infected with *Treponema pallidum.*
Sections showing *Treponema pallidum* and other Spirochaetes.
Cultures of *Spironema Duttoni, Leptospira icterohaemorrhagica* and *icteroides.*
Sections showing *Guanieri* bodies.
Sections showing *Negri* bodies.
Sections showing *Rickettsia* bodies.

XLVIII. Continue the Study of Anaerobes.

C. Examine the beef heart cultures inoculated with the isolated colonies. If spores are present heat 0.5 c.c.–1 c.c. for 10 minutes and make another series of dilutions in deep liver agar. Test 0.5 c.c. of the beef heart culture for aerobic contamination in peptic digest broth and on petic digest agar slants. Incubate 3–4 days at 37° C.

Pathogenicity tests: 1 c.c. of the culture may be inoculated into the leg muscles of a guinea pig to test for pathogenicity.

Toxicity tests: (*a*) The presence of botulinus toxin is demonstrated by feeding 0.5–1 c.c. of the centrifuged or filtered (Berkefeld V) 10-day culture to each of three mice or guinea pigs. Previous to the feeding one mouse is inoculated intraperitoneally with 0.5 c.c. of type A and one with .5 c.c. of type B antiserum; the third animal serves as a control.

(*b*) The presence of tetanus toxin is demonstrated by (1) injecting subcutaneously .5 c.c. of the centrifuged or filtered (Berkefeld V) 10-day culture into two mice or guinea pigs. Previous to inoculation one mouse or guinea pig is inoculated intraperitoneally with .5 c.c. of tetanus antitoxin (the amount of filtrate and tetanus antitoxin injected will depend on the strength of the reagent). (2) Instead of injecting the filtrate and antitoxin separately they may be mixed in a small test tube, allowed to stand in the dark for from 30 to 60 minutes, and then inoculated subcutaneously into the test animal.

References:

HALL, I. C. Practical methods in the purification of obligate anaerobes. *Jour. Infect. Dis.*, 1920, 27, p. 577.

HALL, I. C. Differentiation and identification of the sporulating anaerobes. *Jour. Infect. Dis.*, 1922, 30, p. 445.

KAHN, M. C. Anaerobic spore-bearing bacteria of human intestine in health and in certain diseases. *Jour. Infect. Dis.*, 1924, 35, p. 423.

XLIX. Examination of Smears Containing Fusiform Bacilli.

Vincent's angina: Smears prepared from the exudate of cases of Vincent's angina will be furnished. The smears are stained with Giemsa stain or by Tilden's method. When fresh material is available the method mentioned under (*b*) will be used. Note the fusi-spirochaetal symbiosis. What is meant by symbiosis?

L. Study of Spirochaetes.

1st day. A. Cristispira and Large Spiral Organism.

Open an oyster or clam and expose the crystalline style. Transfer a drop of the liquor and examine it in hanging drop preparation or in the dark field. Thin smears of the same material are stained after alcohol-ether fixation with dilute gentian violet, Giemsa solution, or methylene blue.

Samples of liquid manure are similarly examined.

B. *Spirochaete buccalis,* sp. *dentium.*

Each student will practice the staining of spiral organisms of the buccal cavity. Thin films are prepared from the gums, teeth, etc., showing pyrrhoea and gingivitis and stained by the negative staining methods (cyanochin) and by Benian's Congo red, Tilden's method, and with Giemsa stain. Fresh preparations are also studied in the dark field.

2d day. C. *Treponema pallidum.*

A rabbit infected with *Treponema pallidum* will be available. Dark field and negative staining preparations are prepared from the exudate which the instructor will collect from one of the indurated testes. Staining methods should also be practiced.

D. Specimens of blood, cultures, or animals infected with *Spironema obermeieri* will furnish material for the study of blood parasitic Spirochaetes. Thick blood smears and dark field preparation should be examined.

E. *Leptospira icteroides* and *ictero hemorrhagica.*

Cultures will be furnished. The various methods already described are employed.

F. A number of students will examine several samples of mud (ponds and bay shore) and slime plugs on water spigots for the presence of *Leptospira pseudoicterogenes.*

Reference:

ZUELZER, M. Die Spirochaeten. *Handbuch d. pathogenen Protozoen.* J. A. Barth, Leipzig, 1925, 3, Lief 11, p. 1627.

LI. Study of Filterable Viruses.

A. Vaccina and Variola. A group of students will anesthetize with 2 per cent cocain both corneas of a rabbit; with a fine needle dipped either in vaccine-lymph or the exudate of a smallpox pustule the *epithelium* of the right cornea is scarified. The left cornea is similarly injured with a sterile needle. After 48 to 72 hours the rabbit is chloroformed; the eyes are enucleated, rinsed in salt solution, and placed in alcoholic bichloride of mercury. (Dissolve 4 gm. $HgCl_2$ in 60 c.c. distilled water, and add this to 30 c.c. of 96 per cent alcohol.) The crater-like elevations on the vaccinated cornea are characteristic for the variola virus. Very small and thin sectors of the infected and non-infected cornea are removed after 3 hours' fixation and rapidly embedded as follows:

96 per cent alcohol, 5 minutes
Absolute alcohol, 5 minutes
Absolute alcohol-chloroform, 5 minutes
Pure chloroform, 5 minutes
Saturated solution of hard paraffin in chloroform at 40° C., 5 minutes.

Embed in paraffin; make sections and mount on slides. Stain with Giemsa solution or Heidenham's iron hematoxylin.

For the latter method proceed as follows: Pass sections through xylol, absolute alcohol, 95 per cent, 70 per cent alcohol to distilled water; place the sections in a solution of 3.5 per cent iron alum for 4 hours. Rinse thoroughly in distilled water; stain for 15 minutes in a ripe solution of haematoxylin (1 gm. haematoxylin in 10 c.c. 90 per cent alcohol and 90 c.c. distilled water, ripened for 4 weeks); wash in distilled water and then differentiate in the iron alum solution by controlling the process under the microscope; finally wash in tap water, pass through alcohol to xylol and mount in balsam. The Guarnieri bodies are seen as small deeply stained bodies in the cytoplasm of the epithelial cells. The students will demonstrate the sections to the class.

References:

Cowdry, E. V. Supravital staining vaccine bodies. *Jour. Exper. Med.*, 1922, 36, p. 667.

Scott, J. M., and Simon, C. E. Diagnosis of smallpox by Paul method. *Am. Jour. Hyg.*, 1923, 3, p. 401.

B. *Epithelioma contagiosa:* Specimens of chicken pox material will be handled in the same manner and demonstrated to the class.

C. *Rabies:* Smears or sections of the central nervous system of various animals showing Negri bodies will be demonstrated or supplied for staining and study. The smears are prepared as follows: Impression films with gray matter of the central nervous system (Ammon's horn, cerebellum, and convolutions around the crucial sulcus) on clean slides are dried in air, fixed for 10 seconds in neutral methyl alcohol to which 0.1 per cent picric acid has been added. The excess fixative is removed by filter paper and smears stained in the following solution:

Saturated methyl alcoholic solution of basic fuchsin	1 c.c.
Saturated methyl alcoholic solution of methylene blue	15 c.c.
Methyl alcohol (absolute)	25 c.c.

The smear is then washed in tap water and blotted with filter paper. The Negri bodies are magenta red with blue granules. (Williams-Sellers, see Park and Williams, p. 635.) The sections are prepared from tissues fixed in Zenkner's solution (8 hours) and by Mallory's eosin-methylene blue method (see Mallory and Wright).

The daybook must show a record of the findings and interpretations.

LII. Study of Rickettsia Bodies.

Sections and smears, ticks and bed bugs showing the intracellular Rickettsia bodies of Rocky Mountain spotted fever and typhus will be demonstrated to the class.

Reference:

Wollbach, S. B. Studies on Rocky Mountain spotted fever. *Jour. Med. Res.* 1919, 41, p. 1.

PART III

PROBLEMS IN EXPERIMENTAL PATHOLOGY AND IMMUNOLOGY

PREPARED BY

B. G. ANDERSON

PART III. PROBLEMS IN EXPERIMENTAL PATHOLOGY AND IMMUNOLOGY

PREPARED BY

B. G. ANDERSON

General Directions

As soon as problems have been assigned, students should see the instructor in charge of the experiment regarding date for submitting a written protocol of procedure and time of beginning the experimental work. The protocol must include (1) outline of procedure based upon reading, (2) list of apparatus, special media, chemicals or dyes, and animals required. Requests for special media or cultures must be submitted at least 4 days before such materials are needed by the worker. *Students must sign slips for all special apparatus and are held responsible for loss or breakage.*

The references given in the outlines for the different experiments are merely to guide the student in his reading. A number of the most important publications are available in the library as bound reprints. *Since these files will save the student a great deal of work, it is earnestly requested that he will treat the collection with the utmost care. It is frequently impossible to replace lost copies.*

Students who have had general bacteriology will start immediately the preparation of assigned problems to be presented to the class during the first few weeks. In making oral reports to the class, bear the following points in mind: (1) Outline briefly and clearly the technic employed. (2) Mention only those difficulties in procedure which are inherent to that particular experiment, not mere personal errors in technic. (3) Tabulate results when possible. (4) Confine discussion to the main points of immunological and bacteriological significance and application. (5) Summarize the problem by itemized points. (6) Remember that detailed charts and tables made 6×6 inches or illustrations from books can be thrown on the screen by the projectoscope and so be seen much better by everyone than when written on the blackboard.

During the first few weeks instructors will demonstrate general methods employed in the various types of animal experimentation.

Students are advised to take notes regarding the order of procedure in each technic. The demonstrations will aid students in their particular problems and if properly understood, the student need not spoil or fail to get results because of faulty personal technic. The following procedures will be shown: (1) intraperitoneal, intravenous, and subcutaneous inoculation of rabbits; (2) ear, carotid, and heart-bleeding of rabbits; (3) intracutaneous tests on guinea pigs; (4) bleeding a person from the arm vein.

Second Week

EXPERIMENT 1. STAINING METHODS FOR DISTINGUISHING DEAD AND LIVING BACTERIA

1. Is débris of vegetable origin stainable by methylene blue?
2. What physical phenomena play a rôle in such differential staining?

References:

Henrici, A. T. Differential counting of living and dead cells of bacteria. *Proc. Soc. Exper. Biol. and Med.*, 1923, 20, pp. 293–294.

Kayser, H. Die Unterscheidung von lebenden und toten Bakterien durch die Farbung. *Centralbl. f. Bakteriol.*, 1912, Abt. I, Orig. 62, pp. 174–176.

Burke, G. S. The differential staining of living and dead spores. *Jour. Infect. Dis.*, 1923, 32, p. 433.

EXPERIMENT 2. DEMONSTRATION OF OPTIMUM P_H OF GROWTH

Use *B. proteus* and *B. pyocyaneus* in plain veal infusion broth between optimum and limiting P_H. Employ the methods described by Schoenholz, P., and Meyer, K. F., Optimum H-ion concentration for typhoid bacilli. *Jour. Infect. Dis.*, 1921, 28, p. 384.

1. Why is phenolphthalein an unsatisfactory indicator for bacteriological work?
2. What physical chemical principle underlies the action of buffer systems?
3. How can the optimum P_H range of an organism be enhanced?

References:

Clark, W. M. *The Determination of Hydrogen-Ions.* Ed. 2; Baltimore, Williams and Wilkins, 1922.

Dernby, K. G., and Avery, O. T. The optimum hydrogen-ion concentration for the growth of pneumococcus. *Jour. Exper. Med.*, 1918, 28, p. 345.

EXPERIMENT 3. DEMONSTRATION OF INVOLUTION FORMS

To a series of tubes each containing 10 c.c. of melted agar, add such amounts of a 10 per cent lithium-chloride solution that the final concentration of the LiCl shall be 1, 2, and 3 per cent. Sterilize the tube in the Arnold sterilizer for ½ hour. Slant the tubes. Inoculate as follows:

Sets of 3 tubes of LiCl agar and 1 agar slant without LiCl, are seeded with *B. prodigiosus, B. coli,* and *Vibro Metchnikovi* respectively.

The cultures are incubated at 37° C. and examined daily.

1. Are involutions, forms of degeneration, or do they form a part of the life-cycle?
2. Which organism is usually diagnosed by demonstrating involution forms?

References:

Mellon, R. R. Characteristics of a peculiar pleomorphic diphtheroid. *Jour. of Bacteriol.*, 1917, 2, pp. 81, 269, 447.

Löhnis and Smith. Life cycles of azotobacter. *Jour. Agr. Res.*, 1923, 23, p. 401.

EXPERIMENT 4. DEMONSTRATION OF LUMINESCENT BACTERIA

Prepare sea water-sea fish agar. Spread with portions of the skin of a fish which has been kept at 22° C. until it luminates strongly. Place a small piece of fresh beef in 3 per cent NaCl water. Cover it with a bell jar, allowing a small portion to project from the solution, and keep at 10° C. To culture the light-bacteria, make up the following media at P_H 7.6. Incubate at 22° C.

3 gms. NaCl	10 gms. gelatin
1 gm. peptone	100 c.c. tap water
0.5 gm. glycerin	

Prepare pure cultures. Demonstrate the photogenic properties to the class by dropping the broth through a long glass tube. Discuss the significance.

1. Is photoluminesence destroyed by freezing with liquid air?
2. Is the glow due to an anabolic or katobolic process?
3. What causes the light of the firefly?

References:

Molish, H. Leuchtende Pflanzen *Lafar, Handbuch der technische Mykologie*, 1904, Erster Band.

Bacterial luminescence. Chem. Abstr., 1923, 17, p. 1491; 1924, 18, p. 1437.

Koch, Alfred. *Mikrobiologisches Praktikum.* Berlin, Julius Springer, 1922, p. 34.

EXPERIMENT 5. MECHANISM OF THE GRAM STAIN

With reference to the action of different mordants and the results obtained by varying the H-ion concentration of the staining fluids:

1. Why do old cultures give a mixed picture with Gram stain?
2. To what class of chemical compounds do the so-called 'mordants' of the Gram stain belong?
3. What bearing has the iso-electric point on this problem?

References:

Stearn, E. W., and Stearn, A. E. Chemical mechanism of bacterial behavior. *Jour. Bacteriol.*, 1924, 9, pp. 463, 479, 491.

Stearn, E. W., and Stearn, A. E. The mechanical behavior of dyes. *Jour. Bacteriol.*, 1923, 8, p. 567.

Third Week

EXPERIMENT 6. BACTERICIDAL ACTION OF NORMAL SERA

The method, described in Zinser, Hopkins and Ottenberg (ed. 2, New York, Macmillan Co., 1921), page 20, is as follows:

The materials in this experiment must be measured with sterile pipettes and place in sterile test tubes, with care to avoid contamination during the process of the experiment.

Reagents—

1. Fresh normal rabbit serum, unheated.
2. V. Metchnikovi—24-hour broth culture diluted 1:500 in sterile salt solution.
3. Sterile salt solution.

(*a*) Place 0.5 c.c. of normal rabbit serum in each of two sterile test tubes and heat one tube for half an hour at 56° C. Then set up the following preparations in sterile plugged test tubes.

1. Heated rabbit serum 0.5 c.c. + culture broth (1–5000) 0.5 c.c.
2. Normal rabbit serum 0.5 c.c. + culture broth (1–5000) 0.5 c.c.
3. Salt solution 0.5 c.c. + culture broth (1–5000) 0.5 c.c.

(*b*) Place the tubes in the incubator for two hours, then pour into each tube agar which has been melted in boiling water and cooled to 42° C. Pour the entire contents of each tube into a sterile Petri dish. When the plates are hardened, place them in the incubator in an inverted position. Observe on the following day.

1. How do you explain the results obtained with the heated serum?
2. What is the relation of complement to bacteriolysis?
3. Are sera from various animal species equally lytic? Are all bacterial species destroyed with equal rapidity?

References:

Sakagami, K. Bactericidal action of normal and infected sera on different microbes. *Lancet*, 1916, 1, p. 127.

Eastwood, A. P. The capillary endothelium in relation to antibodies. *Jour. of Hyg.*, 1923–24, 22, p. 355.

Salis-Cohen, S., and Heist, G. D. Bactericidal action of whole blood of rabbits following inoculations of Pneumococcus bacterins. *Jour. Immunol.*, 1919, 4, p. 147.

EXPERIMENT 7. ONE CELL CULTURE BY THE BURRI METHOD

Use Nigrosin instead of India ink. Consult publications dealing with other methods and report.

1. Is the study of bacterial pleomorphism aided by this technic?
2. What advantages has the Barber technic?

References:

ADELMAN, L. Tuschekulturmethode und Teilungsvorgänge bei Bakterien. Centralbl. f. Bakteriol. Abt. I, Orig., 1922, 88, p. 401.

BARBER, M. A. Pipette method for isolation of single microörganisms. *Philippine Jour. of Sci.*, 1914, 9, p. 307.

HORT, E. C. The cultivation of aerobic bacteria from single cells. *Jour. Hyg.*, 1919, 18, p. 361.

ØRSKOV, J. Method for the isolation of bacteria in pure culture from single cells and procedure for the direct tracing of bacterial growth on a solid medium. *Jour. Bacteriol.*, 1922, 7, p. 537.

MALONE, R. H. A simple apparatus for isolating single organisms. *Jour. Path. and Bacteriol.*, 1918–19, 22, p. 222.

BURRI, R. Dàs Arbeiten mit der einzelnen Bakterienzelle unter mikroskopischen Kontrolle. Kraus and Uhlenhuth, *Handbuch der mikrobiologischen Technik*, 1923, 2, p. 895.

EXPERIMENT 8. MUTATION

Streak *B. coli* mutabile on lactose Endo plates or *B. enteritidis* on arabinose Endo plates. Incubate at 37° C.

1. Does a mutated strain retain the alteration of its biochemical activity?
2. What is an atypical *B. coli?*

References:

EISENBERG. Uber Mutationen bei Bakterien und andern Mikroörganismen. *Ergebnisse der Immunitätsforschung*, 1914, 1, pp. 28–142.

BERGSTRAND, H. On the variation of Bacterium coli. *Jour. Bacteriol.*, 1923, 8, p. 173.

PENFOLD, W. J. Studies in bacterial variation, with special reference to the chemical functions of the members of the Typhoid-coli group. *Jour. Hyg.*, 1911, 11, p. 30.

KLOTZ, OSKAR. Temporary alteration of character of an organism belonging to the colon group. *Jour. Infect. Dis.*, 1906–07, Sup. 2–3, p. 35.

EXPERIMENT 9. FEVER PRODUCTION

Inject various amounts of egg white or milk into the ear vein of a rabbit. Ascertain the normal temperature of the rabbit and keep a chart of subsequent temperature rise.

1. What does fever in the human being or animal indicate?
2. How is the diet of a patient adjusted during fever? Why?
3. Is immunity affected by body temperature? How?

References:

VAUGHAN, V. C. *Protein Split Products.* Philadelphia, Lea and Febiger, 1913, p. 373.

PETERSEN, W. F. Protein Therapy and Non-specific Resistance. New York, Macmillan Co., 1923.

HEATH, O. Clinical interpretation of fever. *Proc. Roy. Soc. Med.,* 1923–24, 17:1–2, Section on Medicine, p. 7.

HEATH, O. Meaning of fever. *Proc. Roy. Soc. Med.,* 1923–24, 17:3, Section on Pathology, p. 1.

EXPERIMENT 10. INFLAMMATION

Shave a rabbit's ear and produce an inflammation by irritating it either by hot water or croton oil. Explain the stages of reaction which occur.

1. Have the capillaries any contractile power?
2. How is pain related to inflammation?

Reference:

MCCALLUM, W. G. *Textbook of Pathology.* Ed. 2; Philadelphia, W. B. Saunders Co., 1920, p. 132.

Fourth Week

EXPERIMENT 11. PREPARATION OF A VACCINE

Prepare and standardize 2 large Blake bottles of antityphoid vaccine. Always use a safety pipette when making agglutinations with living antigen. Remember that the material is highly infectious and that all glassware must be autoclaved after the titer has been read.

The method established in 1917 by G. W. McCoy (U. S. Public Health Service, Hygienic Laboratory *Bulletin,* No. 110, 1917) is as follows:

Culture—

(1) Plate the Rawling culture on at least one set of Endo plates; incubate 24 hours.

(2) Examine colonies and mark at least three which have typical appearances and are large and well separated.

(3) From each colony make a transfer to three tubes: 2 broth tubes; a stab and slant of Russell's double sugar agar. Keep corresponding tubes together; incubate 24 hours.

(4) Examine growth and make macroscopic agglutinations by adding one drop of antityphoid serum of high titer to one broth tube inoculated from each colony. Incubate one-half hour at 37° C.

(5) Make several agar slants from the broth tube corresponding to the one giving best positive agglutination and typical appearance on Russell's medium. Incubate 24 hours at 37° C.

(6) Add three-fourths c.c. of salt solution to each slant; scrape off growth, and collect the emulsion in large test tubest or Erlenmeyer flasks.

(7) From each tube make a heavy smear and stain by the Gram method to determine purity of the emulsion.

Media, etc.—

(1) Make 3 per cent agar + 0.5, to phenolphthalein. (P_H 7.2.)

(2) Sterilize 10 to 30 one-liter bottles with flat sides, plugged with absorbent cotton, by dry heat at 170° C. for 1½ hours.

(3) Put 100 c.c. agar in each bottle, and sterilize in autoclave at 15 pounds pressure for 1 hour.

(4) Let agar harden over-night with bottles flat. Invert and place in incubator on slight slant for 24 to 48 hours.

(5) Make 0.85 per cent NaCl solution; autoclave at 15 pounds pressure 1½ hours. Plant 5 c.c. of the salt solution from each flask in freshly heated and cooled glucose broth fermentation tubes. Incubate 48 hours; discard if contaminated.

(6) Make several absorbent cotton swabs on wire rods; put in test tubes and sterilize by dry heat at 170° C. for 2 hours.

Vaccine—

(1) Inspect each bottle for eivdence of contamination, flame plug, and remove plug and flame mouth of bottle. Remove water of condensation with a sterile pipette.

(2) Inoculate each bottle twice with well-charged swab from a tube of emulsion. Invert on slant and incubate 24 hours. Make plate cultures from emulsion which is left. (Inoculation may be done by placing a thin layer of the emulsion upon the agar and flowing it evenly over the surface.)

(3) Examine each bottle with great care and discard any bottles which have growths not typical of a typhoid culture or which show Gram positive organisms on staining. This is a most important step in the procedure because if any spore-bearing organisms are passed over and get into the vaccine they will not be killed by subsequent measures and the vaccine will not be sterile.

(4) Add 30 to 50 c.c. of salt solution to each bottle; place on slight slant, so that salt solution will moisten growth for few minutes. Wash off growth by holding the flask in the hands and slightly tilting the bottle from side to side so that the fluid flows across the surface of the agar. Collect the emulsion in 500 c.c. flasks.

(5) Mix collections, measure emulsion, and take sample for counting.

(6) Pour emulsion into 500 c.c. flasks, put tin foil on plug, weight with lead around neck, and put in water bath. A control flask with salt solution and thermometer is put in bath. Another thermometer is suspended in the bath direct. The water should come above the fluid in the flasks by 1 to 2 inches.

(7) Heat emulsion at 53° C. to 54° C. in well-agitated water bath, watched constantly to keep the temperature accurate. Remove flask after one hour at this temperature.

(8) The neck of the flask should be thoroughly flamed to a point below the level heated by the water bath.

(9) From the count estimate amount of salt solution to be used to give 1,000,000,000 bacilli per cubic centimeter and add trikresol to diluting fluid, so that the final strength of trikresol will be 0.4 per cent. Dilute vaccine according to count in large sterile bottles. Trikresol should be entirely in solution before adding to vaccine. Its solution is more prompt if added to warm salt solution and shaken during the addition.

(10) After 12 to 24 hours at room temperature take sample for culture.

Counting—

(1) A well-mixed sample of the emulsion is placed in a small sterile conical glass.

(2) A portion of this is diluted in a red-blood cell counting pipette using the following solution as a diluent: 5 per cent phenol solution, 100 c.c.; Ziehl-Neelsen carbol-fuchsin, 0.25 c.c. Mix and let stand 10 minutes and filter. This diluting fluid kills the typhoid bacilli and stains them light red.

(3) Count the number of bacteria in a Helber counting chamber in a manner similar to counting blood cells.

(4) Estimate the number of bacteria per cubic millimeter of emulsion. (The Helber counting chamber is 0.02 mm. deep, the blood counting chamber 0.1 mm. Hence the capacity of the Helber chamber is one-fifth that of the blood-counting chamber.)

Tests—

(1) Plate 2 loopfuls of emulsion left from inoculation on 2 sets of plates.

(2) Plate 0.1 c.c. heated vaccine on each of 3 plates. These plates should show some colonies of typhoid bacilli, but of no others.

(3) Plant cresolized vaccine in at least 6 anaerobic tubes, of freshly heated and cooled glucose broth, 5 drops each in 3 tubes and 20 drops each in 3 others. These tubes should be sterile; if they are not, the vaccine must be discarded. Incubate 7 days.

(4) If the foregoing tests are satisfactory at end of 48 hours, proceed with the following tests. Inoculate 2 guinea pigs, 1.5 c.c. intraperitoneally; 2 white mice, 0.5 c.c. subcutaneously. Give each of 3 rabbits, 0.5 c.c.; after 3 days, 1 c.c.; after 6 days, 1 c.c.; all subcutaneously. Agglutination is tested after 5 days and should equal the controls.

Final agglutination should run at least to 1–500 dilution. The mice and the guinea pigs are used as a test for tetanus. The mice also serve as a check on the amount of preservative. The rabbits are used as a test for agglutinin production.

Filling of ampules—

(1) Any apparatus that reduces the possibility of contamination to a minimum may be used.

(2) Care must be taken to see that cracked ampules are excluded and that the seal is perfect. To do this, place the filled sealed ampules in a water bath at 40° C.; stir gently to insure uniform heating to this temperature. Remove the ampules to an iced water bath to which sufficient methylene blue is added to give it a deep blue color. After sufficient time has elapsed to allow all ampules to be cooled, remove the ampules and rinse in tap water. The contents of defective ampules will be colored blue.

Test of finished product—

(1) At least 3 ampules, selected at random from each lot of the finished product, are examined, or 3 per cent if more than 100 are filled at one time, up to 20 ampules for each lot. These are tested for sterility by planting from each ampule 5 drops in a Smith fermentation tube, and 20 drops in a similar tube, containing about 30 c.c. of recently heated and cooled glucose broth having a reaction of + 0.5 per cent to phenophthalein at 100° C.

(2) The tubes are inoculated and examined after 2, 4, and 7 days; they should be agtitated after each examination and the lowest part of the bend should be examined for slight growth before agitation.

(3) If growth is found in any tube, a second test is made, using double the number of ampules used in the first test.

(4) If the second test shows growth, the vaccine is discarded.

1. Can you use your own blood for a Wright count if you have been recently vaccinated against typhoid?
2. What kind of media and equipment do you need for a sputum vaccine? For acne vaccine?
3. Why is an autogenous vaccine to be preferred to a stock preparation?

References:

Glynn, E., Powell, M., Rees, A. A., and Cox, G. L. Comparison of Wright, haemocytometer and plate culture methods of vaccine standardization. *Jour. Path. and Bacteriol.*, 1913, 18, pp. 379–400.

Hopkins, J. G. Methods of standardizing bacterial vaccines. Jour. Amer. Med. Assoc., 1913, 60, p. 1615.

McCoy, G. W., and Bengston, I. A. Deterioration of typhoid vaccine. U. S. Hygienic Laboratory Bulletin, No. 122, 1920.

EXPERIMENT 12. CUTI-INFECTION AND CUTI-IMMUNITY IN ANTHRAX

Immunize four guinea pigs by treating the skin with 2d vaccine. Inject subcutaneously with ½ c.c. of virulent culture. Inject 2 guinea pigs intraperitoneally with ½ c.c. of culture without infecting the skin.

1. Discuss other phases of local or tissue immunity.
2. What other methods are used to lessen the virulence of biological material?

References:

Besredka, A. Charbon. Ann d. l'Institut Pasteur, 1921, 35, p. 421.

Brocq-Rousseu et Urbain. Vaccination du cheval contre le charbon bactéridien, par le voie cutanée. *Ann.* d. l'Institut Pasteur, 1924, 38, p. 268.

Fifth Week

EXPERIMENT 13. PFEIFFER'S PHENOMENA IN VIVO

Immunize a rabbit with *V. Metchnikovi* by the Gay-Fitzgerald method. (See experiment of the sixth week.) A 1:4000 titer is of ample strength for demonstrating bacteriolysis. Bleed the rabbit from the heart when the desired titer has been reached. Inactivate a portion of both immune and normal rabbit sera at 56° C. for half an hour. Prepare suspensions of 12-hour agar slant of *B. coli* and *V. Metchnikovi* emulsified in 10 c.c. of saline. The lytic phenomena are to be demonstrated by intraperitoneal injection of guinea pigs with various combinations of culture and sera. Until the student is familiar with the technic only one animal should be handled at a time.

The initial injection is done with a Luer syringe; great care must be taken to avoid perforating the intestine. Subsequently samples are removed from the cavity at 5, 10, 20, 40, and 60-minute intervals by means of a capillary Pasteur pipette. Capillarity will usually cause sufficient fluid for examination to rise in the pipette without application of suction. Cover the puncture each time with a piece of cotton soaked in lysol. First, several smears of the peritoneal fluid are made for subsequent staining, then the specimen is studied for loss of motility, granulation, etc., in hanging drop preparations. The smears are fixed in a mixture of equal parts of ether and alcohol and stained for half an hour with carbol thionin or dilute carbol fuchsin. If leucocytes appear in the specimen, an eosin counterstain may be used. The carbol thionin is decolorized by rapid passage through graded alcohols (40 per cent to 90 per cent) and the smear is counterstained with eosin.

Three guinea pigs are injected intraperitoneally with 4 c.c. of the following material:

Pig. 1. Mix equal parts of *V. Metchnikovi* suspension with 1:10 saline dilution of inactivated immune serum.

Pig. 2. Mix equal parts of *V. Metchnikovi* suspension with 1:10 saline dilution of normal inactivated rabbit serum.

Pig 3. Mix equal parts of *B. coli* suspension with 1:10 saline dilution of inactivated immune serum.

1. Differentiate between the processes of agglutination, bacteriolysis, and hemolysis.

2. Is the technic practical for the diagnosis of cholera?
3. What is the significance of the normal serum and the *B. coli* controls used in the procedure outlined?

References:

Morrison, L. F. On the origin of alexin (complement) in the guinea-pig blood. *Jour. Immunol.*, 1922, 7, p. 435.

Friedberger, E. Technik und Wesen der Pfeifferischen Phenomens, *in* Abderhalden, *Handbuch der biologischen Arbeitsmethoden.* Abt. 13, Teil 2, Heft 2, p. 16.

Bordet, J., and Gay, F. P. *Studies in Immunity.* New York, John Wiley, 1909, p. 56.

EXPERIMENT 14. PFEIFFER'S PHENOMENA IN VITRO

Immunize a rabbit with *V. cholerae* using the method of Harriette Chick (see general references, experiments of sixth week). Bleed the rabbit from the heart when the titer has reached 1:4000. Inactivate immune and normal rabbit sera at 56° C. for half an hour. Bleed a guinea pig from the heart and make a 1:50 dilution of this fresh serum for complement. Keep all reagents sterile; set up and incubate the following series of tubes using every precaution against external contamination.

Place 1 c.c. of the complement dilution in 10 Wasserman tubes. Rub into the fluid of each tube one loopful of the centrifugalized 12-hour culture grown in veal infusion broth P_H 7.8. An emulsion of living organisms may be more accurately controlled by measuring 0.1 c.c. of a suspension of standard opacity with a safety pipette.

Add to individual tubes 1 c.c. of a known dilution of immune or normal serum. The five final dilutions of immune serum should range between 1:100 and 1:10,000, the normal serum between 0 and 1:100. Set up controls for sterility of reagents and specificity of the reaction. Shake well and incubate at 37° C. At the end of the first hour examine hanging drop preparations of each tube. Stains should be prepared of each dilution as in the intraperitoneal method of demonstrating bacteriolysis. Remember the material is highly infectious and autoclave all slides after use. Smears may be fixed in 10 per cent formaldehyde.

After the tubes have been incubated for two hours, the extent of the lysis can be determined by plate culture. Convey two loopfuls from each serum dilution tube into melted agar (42–45° C.) and plate out in the usual method. Plate the culture and the reagent controls for comparison.

1. What advantages has this method over the *in vivo* procedure?
2. Why is fresh guinea pig serum necessary?

EXPERIMENT 15. EFFECT OF SALT ON AGGLUTINATION

Use technic of Zinsser *et al.* (ed. 2, New York, Macmillan Co., 1921), p. 72, as follows:

REAGENTS—

1. Serum of animal immunized to *B. typhosus.*
2. *B. typhosus*—24-hour agar slant.
3. Distilled water.
4. 10 per cent salt solution.
5. Copper sulphate solution 0.8 per cent.

Prepare typhoid suspension (killed with formalin as in previous lesson for the sake of safety). Place in each of two centrifuge tubes, with pointed tip, 2 c.c. of the suspension. To tube A add 2 c.c. of agglutinating serum diluted 1–50. To tube B add 2 c.c. of distilled water. Allow the tubes to stand at 37° C. for 30 minutes. Centrifugalize the tubes at high speed until the supernatant fluid is clear. Resuspend the sediments in 5 c.c. of distilled water and again centrifugalize. To the washed sediments add 2 c.c. of distilled water and draw the mixture repeatedly in and out of the capillary pipette in order to break up the clumps and obtain an even suspension. Set up the following tests in agglutination tubes:

1. Sediment A 0.5 c.c.	Distilled water 0.5 c.c.		
2. Sediment A 0.5 c.c.	10 per cent salt sol. 0.09 c.c., dist. water 0.4 c.c.		
		Copper sulphate sol. 0.8%	Dist. water
3. Sediment A 0.5 c.c.		0.02 c.c.	0.5 c.c.
4. Sediment B 0.5 c.c.		0.02 c.c.	0.5 c.c.
5. Sediment B 0.5 c.c.		0.1 c.c.	0.5 c.c.
6. Sediment B 0.5 c.c.		———	0.5 c.c.

The tubes are placed in the water bath at 37° C. for one hour and then observed. Tubes 2, 3, and 5 should show agglutination.

1. Has Ehrlich's theory of agglutination been upheld by later investigators?

References:

FIELD, C. W., AND TEAGUE, O. On the electrical charge of the native proteins and the agglutinins. *Jour. Exper. Med.,* 1907, 9, p. 222.

BUCHANAN, R. E. Agglutination. *Jour. Bacteriol.,* 1919, 4, p. 73.

ARKWRIGHT, J. A. Variation in bacteria in relation to agglutination, both by salts and by specific serum. *Jour. Path. and Bacteriol.,* 1921, 24, p. 36.

NORTHROP, J. H. Stability of bacterial suspensions. *Jour. Gen. Physiol.,* 1922–23, 5, p. 605.

WINSLOW, C. E. A., FALK, I. S., AND CAULFIELD, M. F. Electrophoresis of bacteria as influenced by P_H and presence of sodium and calcium salts. *Jour. Gen. Physiol.,* 1923, 6, p. 177.

EXPERIMENT 16. ACID AGGLUTINATION

Six volumetric flasks (100 c.c.) are used to make up the following mixtures of normal acetic acid and normal sodium hydroxide of known H-ion concentration. Add distilled water to make up a total volume of 100 c.c.

		a	*b*	*c*	*d*	*e*	*f*
N–NaOH	c.c.	5	5	5	5	5	5
N–CH_3COOH	c.c.	7.5	10	15	25	45	85
P_h		5.0	4.7	4.4	4.1	3.8	3.5

Plant several agar slants of both *B. typhosus* and *B. paratyphosus* A and B; incubate for 24 hours. Wash off the growth on each slant with 20 c.c. distilled water. Place 3 c.c. of the suspension of each organism in 6 test tubes. Add 1 c.c. of the acetic acid series to a tube labeled with the corresponding P_H. Incubate and observe every 15 minutes without shaking. As soon as tube "C" or either of the adjacent tubes shows a flocculation, place the series at room temperature. After about 2 hours at this temperature a definite end point will be reached which will not change for a day or more.

1. Why is it important to know exactly where acid agglutination begins?

2. Does this optimum P_H of agglutination aid in differentiating organisms?

References:

Michaelis, L. Die Säureagglutination der Bakterien. *Deutsch. med.* Wochschr., 1911, 37, p. 969.

Hirsch, E. F. Hydrogen ion changes in agglutination of bacteria by immune sera. *Jour. Infect. Dis.*, 1922, 30, p. 651.

de Kruif, Paul. Change of acid agglutination as index of bacterial mutation. *Jour. Gen. Physiol.*, 1922, 4, p. 387.

Gouwens, W. E. Acid agglutination of paratyphoid bacilli. *Jour. Infect. Dis.*, 1923, 33, p. 113.

Shibley, G. S. Studies in agglutination. *Jour. Exper. Med.*, 1924, 39, p. 245.

Michaelis, L. Technik der Säureagglutination, *in* Abderhalden, E., *Handbuch der biologischen Arbeitsmethoden*, Abt. 13, Teil 2, Heft 2, p. 287.

EXPERIMENT 17. PROTECTION EXPERIMENT

Mice or Guinea Pigs against *B. aertryke*

To test the immunity, inject living culture, subcutaneously, intraperitoneally, and by mouth; the latter after an initial bile feeding.

1. Discuss various kinds of local and tissue immunity.
2. What is the effect of bile ingestion?

References:

Besredka, A. De la vaccination contre les états typhoïdes par la voie buccale. Ann. d. l'Institut Pasteur, 1919, 33, pp. 301, 882.

Webster, L. T. Experiments on normal and immune mice with a bacillus of mouse typhoid. *Jour. Exper. Med.*, 1922, 36, pp. 71, 97.

Clark, P. F., Zellmner, C. E., and Stone, H. W. Observations in non-specific immunity. *Jour. Infect. Dis.*, 1922, 31, p. 215.

Nichols, H J., and Stimmel, C. O. Protection against typhoid and like infections by vaccination. *Jour. Exper. Med.*, 1923, 38, p. 283.

Sixth Week

A. ARTIFICIAL ANTIBODY PRODUCTION

For agglutination use the modified Dreyer technic, page 123.

Reference:

Locke, E. A rapid method of producing agglutins. *Univ. Calif. Publ. Path.*, 1912, 2, p. 91, quotation as follows:

B. typhosus

Rabbit number	Emulsion dosage	Number of bacteria	Bled on the 8th day	Bled on the 12th day
17	0.1 c.c.	60,000,000	Titer 1:2000	Titer 1:4000
18	0.1 c.c.	60,000,000	Titer 1:80,000	Titer 1:40,000
19	0.1 c.c.	60,000,000	Titer 1:2000	Titer 1:4000

V. cholerae asiaticae

20	0.1 c.c.	85,000,000	Titer 1:400	Titer 1:2000
21	0.1 c.c.	85,000,000	Titer 1:400	Titer 1:2000
22	0.1 c.c.	85,000,000	Titer 1:200	Titer 1:400

Author's Note: All injections are intravenous with killed vaccines. The daily dose of bacteria has been increased in this laboratory to three billion organisms since this work was published. Judgment must be used about the size of the initial injection of some toxic cultures. If the first series of injections does not yield a sufficiently high titer, a second series is begun in the same manner.

TABLE 12

The Preparation and Use of Certain Agglutinating Sera

By Harriette Chick, C.Sc.

From *Lancet*, 1916, 1, p. 857

Table Giving Preparation and Properties of Agglutinating Sera of the Different Pathogenic Intestinal Organisms

Note.—(The doses are arranged for rabbits of approximately 2000 gm. weight.) Intravenous injections throughout. The numbers expressing titre refer to the highest dilution in which macroscopic agglutination is clearly visible to the naked eye under the conditions of the test. The specificity tests in every case were made with the serum obtained at the final bleeding.

Organism	Day	Dose	Treatment of vaccine	Titre with homologous organism	Specificity		Method of test
					Organism	Titre	
B. typhosus. Strain: "Simpson."	1	1000 millions (1/20–1/30 slope).	Heated ½ hour at 57° C.		B. parat. B.	About= 500	Macroscopic, 2 hours, 37° C.
					B. ent. Gärtner.	About=1000	
	6	2000 millions (1/10 slope).	Not heated.		B. parat. A.	Less than 125	
	13	If titre is high, bleed if not, give 10,000 millions (1/2 slope).	Not heated.	8000 (probably)	B. dys. Y.	Less than 125	
					B. dys. Shiga.	Less than 125	
					V. cholerae.	Less than 125	
	20	Bleed.		8000–16,000			
B. typhosus. Strain: "Chapman."	Immunisation as above.			4000–8000	B. parat. B.	Less than 250	Macroscopic, 2 hours, 37° C.
					B. ent. Gärtner.	Less than 250	
					B. parat. A.	Less than 125	
*B. typhosus. Strain: "Guy."	Immunisation as above.			4000–8000	B. parat. B.	About=1000	Macroscopic, 2 hours, 37° C.
					B. ent. Gärtner.	Less than 250	
					B. parat. A.	Less than 125	
*B. typhosus. Strain: "Horam."	Immunisation as above.			32,000	B. parat. A.	Less than 125	Macroscopic, 2 hours, 37° C.
					B. parat. B.	Less than 1000	
					B. ent. Gärtner.	2000–4000	

* Considered unsuitable for distribution.

TABLE 12—(*Continued*)

Organism	Day	Dose	Treatment of vaccine	Titre with homologous organism	Specificity		Method of test
					Organism	Titre	
B. paratyphosus B. Strains: "Schottmüller-Bainbridge."	1	150 millions.	Heated ½ hour at 57° C.		B. typhosus.	Less than 250	Macroscopic, 2 hours, 37° C.
			Heated ½ hour at 57° C.		B. parat. A.	Less than 500	
"McWeeney," or "McNee 9."	6	300–400 millions.	Heated ½ hour at 57° C.		B. ent. Gärtner.	Less than 250	
	12	1200 millions.	Heated ½ hour at 57° C.		B. dys. Y.	Less than 125	
	19	If titre is high, bleed; if not, give 5000 millions (about 1/6 slope).	Heated ½ hour at 57° C.	8000 (probably)	B. dys. Shiga.	Less than 125	
					V. cholerae.	Less than 125	
	26, 27	Bleed.		8000–16,000			
B. paratyphosus A. Strains: "Schottmüller, original," "Oxford," or "Haddon."	1	100 millions.	Not heated.		B. typhosus.	Less than 500	Macroscopic, 2 hours, 37° C.
	6	400 millions.	Not heated.		B. parat. B.	Less than 500	
	11	1200 millions.	Not heated.		B. ent. Gärtner.	Less than 500	
	16	4000 millions (about 1/5–1/6 slope).	Not heated.		B. dys. Y.	Less than 125	
					B. dys. Shiga.	Less than 125	
	23, 24	Bleed.		8000–16,000	V. cholerae.	Less than 125	
B. enteriditis Gärtner. Strain: "D. H. Bainbridge."	Immunisation as above for B. paratyphosus A.			16,000–32,000	B. typhosus.	Less than 50	Macroscopic, 2 hours, 37° C.
					B. parat. A.	Less than 500	
					B. parat. B.	About=1000	
					B. dys. Y.	Less than 125	
					B. dys. Shiga.	Less than 125	
					V. cholerae.	Less than 125	
B. dysenteriae Flexner. Strain: "Elstree."	1	3000–5000 millions (about 1/10 slope).	Heated ½ hour at 57° C.		B. typhosus.	Less than 125	Macroscopic, 4 hours, 37° C.
					B. parat. A.	Less than 125	
	6–9 (according to condition of animal)	6000–10,000 millions (about 1/4 slope).	Heated ½ hour at 57° C.		B. parat. B.	Less than 125	
					B. ent. Gärtner.	Less than 125	
					B. dys. Y.	=1000–2000	
					B. dys. Shiga.	Less than 250	
	15–18	Bleed.		4000 (rarely 8000)			

TABLE 12—(*Concluded*)

Organism	Day	Dose	Treatment of vaccine	Titre with homologous organism	Specificity		Method of test
					Organism	Titre	
B. dysenteriae Y. Strain: "Hiss and Russell."	As above	for B. dys. Flexner; in some case used th roughout with good results.	s unheated vaccines were	2000–4000 (rarely 8000)	B. typhosus. B. parat. A. B. parat. B. B. ent. Gärtner. B. dys. Flexner. B. dys. Shiga.	Less than 125 Less than 125 Less than 125 Less than 125 =2000–4000 Less than 500	Macroscopic, 4 hours, 37° C.
V. cholerae.	1	1000 millions.	Heated ½ hour at 57° C.		B. typhosus. B. parat. A.	Less than 125 Less than 125	Macroscopic, 2 hours, 37° C.
	6	3000 millions.	Heated ½ hour at 57° C.		B. parat. B.	Less than 125	
	11	10,000 millions (about 1/10 slope).	Heated ½ hour at 57° C.		B. ent. Gärtner. B. dys. Y.	Less than 125 Less than 125	
	16	20,000 millions (about 1/5 slope).	Heated ½ hour at 57° C.		B. dys. Shiga.	Less than 125	
	23	If titre is high, bleed; if not, give 50,000 millions (1/2 slope).	Heated ½ hour at 57° C.	4000–8000			
	31, 32	Bleed.		4000–8000			
M. melitensis. Strain: "Bassett-Smith."	1	2000 millions.	Heated ½ hour at 57° C.		M. paramelitensis.	500	Macroscopic, 4 hours, 37° C.
	8	4000 millions.	Heated ½ hour at 57° C.				
	16	Bleed.		6000			
M. paramelitensis. Strain: "Br."	1	2000 millions.	Heated ½ hour at 57° C.		M. melitensis.	Less than 125	Macroscopic, 4 hours, 37° C.
	8	4000 millions.	Heated ½ hour at 57° C.				
	15	10,000 millions.	Heated ½ hour at 57° C.				
	23	Bleed.		1000			

References:

BULL, CARROLL G. The influence of typhoid bacilli on the antibodies of normal and immune rabbits. *Jour. Exper. Med.*, 1916, 23, p. 419, quotation as follows:

Immunization of animals.—Rabbits were immunized to typhoid bacilli as follows: 1st day, 0.1 of an agar slant of heat-killed bacilli was injected into the peritoneum; 2d day, 0.1 of an agar slant of heat-killed bacili was injected into the vein; 5th day, 0.1 of an agar slant of living bacilli was injected into the vein; 7th and 9th days, repetition of the 5th day treatment. Ten days after the last injection the sera gave agglutination reactions in dilutions varying from 1:30,000 to 1:200,000.

HARVEY, W. F. Production of high titer sera. *Indian Jour. of Med. Res.*, 1919–20, 7, p. 694, quotation as follows:

Conclusions

1. A convenient series of doses for the intravenous inoculation of goats with living organisms is that number of organisms corresponding to 1, 2, 3, 4 mg. of dried bacterial substance. Fowls can stand large doses intravenously.
2. No harm results from frequent repetition of intravenous inoculations in goats or fowls.
3. A 7 or 10-day interval is a convenient interval to allow between inoculations and may be adopted in goats and fowls without reference to restoration of body weight.
4. Intravenous inoculation of antigen—as is now well known—is more efficient for the production of antisera than intraperitoneal. Subcutaneous inoculations are not satisfactory except from the point of view of lessened fatality.
5. Very large doses of living organisms such as the staphylococcus or cholera may be given intravenously to goats. Organisms of the dysentery group have to be more carefully administered than others.
6. Suspensions of living organisms obtained from laboratory strains may be given *ab initio* intravenously in goats.
7. Good antisera are obtainable by confining oneself to intravenous inoculation of suspensions of killed organisms.
8. Organisms simply desiccated, form a very satisfactory, very stable, and very efficient antigen.
9. Rabbits yield a serum of higher titer and higher specificity than goats. Goats are exceedingly convenient animals for the production of high titer sera and yield far larger amounts of serum than rabbits yield. Goat serum can be procured in such large quantities that it may be issued undiluted. Fowls have certain advantages over both goats and rabbits.
10. The titer of an agglutinating serum once raised can be very easily maintained in goats by subcutaneously inoculation.

GRÜBER AND DURHAM. *Münich med. Wochschr.*, 1896.

GAY, F. P., AND FITZGERALD, J. G. An improved rapid method of producing antisera, precipitins, and hemolysins. *Univ. Calif. Publ. Path.*, 1912, 2, p. 77.

HINE, T. G. M. Notes on rapid preparation of high titer agglutinating serum for meningococcus. *Jour. Roy. Army Med. Corp.*, 1915, 25, pp. 424, 693.

SANDS, J. E. A comparative study of methods for the preparation of typhoid agglutinogens. *Jour. Immunol.*, 1920, 5, p. 97.

EASTWOOD, A. Capillary endothelial beds in relation to antibodies. *Jour. Hyg.*, 1923–24, 22, p. 355.

EXPERIMENT 18. PRODUCTION OF B. TYPHOSUS ANTISERUM

Standardize antigen by plate method.

1. What are the advantages and disadvantages of standardization by plate count?

EXPERIMENT 19. PRODUCTION OF B. PARATYPHOSUS A. ANTISERUM

Immunize 2 rabibts against *B. paratyphosus* A. Standardize antigen by haemocytometer.

1. What is the chemical nature of antigens?
2. What rules should be followed in the observation of the physical condition of animals?

EXPERIMENT 20. PRODUCTION OF B. PARATYPHOSUS B. ANTISERUM

Immunize 2 rabbits against *B. paratyphosus* B. Standardize antigen by haemocytometer.

1. At what temperature are agglutinins destroyed?
2. Does heating the serum effect the coagglutinins?

EXPERIMENT 21. POLYVALENT B. TYPHOSUS–B. PARATYPHOSUS B ANTISERUM

Immunize 2 animals against *B. typhosus* and *B. paratyphosus* B simultaneously. Standardize both antigens separately by Hopkins tube. Check the rate of the centrifuge by the revolution counter.

1. Does the agglutinin titer against the different members of the typhoid-paratyphoid rise with equal rapidity?

EXPERIMENT 22. PRODUCTION OF B. DYSENTERIAE ANTISERUM

Immunize 2 rabbits against *B. dysenteriae Shiga.* Standardize antigen by Wright count. Proceed carefully as culture may be toxic.

1. By what means may the toxicity of a culture be reduced?
2. How may the reaction of the animal be made less severe?

Reference:

FLEXNER, S., AND AMOSS, H. L. Rapid production of antidysentery sera. *Jour. Exper. Med.*, 1915, 21, p. 515.

EXPERIMENT 23. PRODUCTION OF B. ENTERIDITIS ANTISERUM

Immunize 2 rabbits against *B. enteriditis.* Standardize antigen by Wright method.

1. What constitutes a good antiserum?
2. How may antisera be preserved?

Sixth Week

B. PHENOMENA OF COAGGLUTINATION

General directions and references—

Use modified Dreyer agglutination technic. Make up standard antigens for agglutination to obtain high degree of specificity according to Army Medical School technic found in Stitt, ed. 7, p. 244. Prepare 2 one-liter flasks of broth antigen from the organism corresponding to the antisera with which the individual problem deals. Run sterility tests on bulk antigen, then divide at least one liter into 250 c.c. sterile flasks for exchange with others doing similar experiments. Prepare absorbing antigen by seeding 2 Blake bottles of peptic digest agar with each organism which is needed, using *B. coli* for a control. Incubate 24 hours and wash off the heavy growth in each bottle with saline containing 0.5 per cent formalin. Place each type of organisms in a small flask and keep on ice for 5 days. Shake the emulsion daily and at the end of the period test for sterility. Determine the titer of the antiserum with the various antigens to ascertain the degree of coagglutination.

The antiserum to be absorbed is diluted 1:10 with saline. Five c.c. of this dilution are placed in sterile centrifuge tubes and an equal quantity of the absorbing antigen added to each respective tube, making a 1:20 dilution. Two controls are necessary. The effect of incubation and centrifugalization on the titer without the presence of organism is determined. Another tube of serum dilution should be absorbed with a non-specific organism such as *B. coli.* The tubes are incubated for 2 hours, left in the ice chest over-night, and then centrifugalized for one hour. The clear serum is decanted and a preliminary test made with the absorbing strain to determine whether the agglutinins have been completely removed. If not, the serum is reabsorbed, using packed bacterial cells for the antigen in order not to alter the 1:20 dilution. The following day the entire titration of the various absorbed antisera is done. Account for the alternations in the original titer.

References:

Castellani, A. Die Agglutination bei gemischter Infection und die Diagnose der letzteren. *Ztschr. f. Hyg.,* 1902, 40, p. 1.

Andrewes, F. W., and Inman, A. C. Study of serological races of the Flexner group of dysentery bacilli. Med. Res. Council, Special Report Series No. 42, 1919.

FEUSIER, M. L., AND MEYER, K. F. Principles in serologic grouping of B. abortus and B. melitensis. *Jour. Infect. Dis.*, 1920, 27, p. 185.

ANDREWES, F. W. Studies on group agglutination. *Jour. Path. and Bacteriol.*, 1922, 25, p. 505.

KRUMWIEDE, C., COOPER, G., AND PROVOST, D. J. Agglutininabsorption. *Jour. Immunol.*, 1925, 10, p. 55.

EXPERIMENT 24. ABSORPTION OF B. PARATYPHOSUS B ANTISERUM

Absorb serum obtained in Experiment 20. Read recent literature pertaining to specificity of antisera from colon-typhoid group of organisms.

1. Discuss the relation of the animal typhoid organisms to the *B. paratyphosus* B. group.
2. Are all species of animals likely to give non-specific antisera?

EXPERIMENT 25. ABSORPTION OF B. TYPHOSUS ANTISERUM

Absorb sera obtained in Experiment 18. Read literature relating to cross-agglutination in the *B. dysenteriae* group of organisms.

1. What is meant by non-specific absorption?
2. Why is agglutination often absent in the lower serum dilutions?

References:

OLITSKY, P. K. Experimental study of vaccination against bacilli dysenteriae. *Jour. Exper. Med.*, 1918, 28, p. 69.

MURRAY. Attempted classification of 53 strains of bacillus dysentery. *Jour. Roy. Army Med. Corps.*, 1918, 31, p. 353.

GARDNER, A. D. The agglutination of standard agglutinable cultures of the Flexner-group of dysentery bacilli by non-dysenteric human sera. *Jour. Hyg.*, 1923, 21, p. 399.

EXPERIMENT 26. ABSORPTION OF A POLYVALENT ANTISERUM

Absorb sera obtained in Experiment 21. Read literature pertaining to serological grouping among streptococcus organisms. Make agglutinating antigen for *B. enteriditis* only.

1. Summarize the difficulties encountered in streptococcus agglutination.
2. How do the various methods of immunization affect the specificity of antisera?

References:

Dochez, A. R., Avery, O. T., and Lancefield, R. C. Studies on the biology of Streptococcus. I. Antigenic relationships between strains of Streptococcus haemolyticus. *Jour. Exper. Med.*, 1919, 30, p. 179.

Tunnicliff, R. Studies on the specificity of streptococci. *Jour. Am. Med. Assoc.*, 1920, 75, p. 1339.

Shibley, G. S. Agglutination of streptococci. *Jour. Exper. Med.*, 1924, 39, p. 245.

Sherman, L., and Dochez, A. R. *Proc. Soc. Exper. Biol. and Med.*, 1924, 21, p. 184.

Stevens, F. A., and Dochez, A. R. Studies on biology of Streptococcus. *Jour. Exper. Med.*, 1924, 40, p. 253.

EXPERIMENT 27. INTESTINAL FLORA

Study the normal intestinal flora of 6 rats when fed upon bread and green vegetables. Divide them into 3 groups and study the bacterial changes caused by feeding each rat the following daily rations:

(1) About 12 gms. bread to which 3 gms. of dry lactose are added.

(2) About 12 gms. bread and either sterile milk or milk soured with *B. acidophilus.*

(3) Cooked meat (10–15 gms. daily) with a little bread.

TECHNIC FOR THE EXAMINATION OF A STOOL SPECIMEN

Morris, Porter and Meyer. *Journal of Infectious Diseases*, 1919, 25, p. 349.

1. Preparation of the 'Stock Dilution':

(1) Weighing: weigh 500 mg. of stool on a sterile watch-glass.

(2) Emulsifying: wash thoroughly into a sterile mortar with 10 c.c. of sterile salt solution; rub with pestle until a homogeneous emulsion, free from visible clumps, is obtained.

(3) Dilution: transfer to a small Erlenmeyer flask and dilute with salt solution to a volume of 50 c.c. Each c.c. of this stock solution represents an equivalent of 10 mg. of fecal material. Fluid stools are emulsified in salt solution and standardized to the same density as one of the known weighed preparations.

2. Microscopic Examination and Differential Count:

   (1) Prepare smear from the 'stock dilution' and stain by Gram's method.

   (2) With the aid of a squared field ocular make a direct count of at least two fields of the following:

      (*a*) Percentages of gram-negative organisms.

      (*b*) Percentages of gram-positive organisms.

      (*c*) Percentages of gram-positive rods.

      (*d*) Percentages of gram-positive cocci.

3. Plating Procedures:

   (1) 'Dalli' one standard loop of stock dilution over the surface of one or two carefully dried Endo plates (Robinson's and Rettger's modification).

   (2) Sugar-free veal or beef liver-infusion-Difco-peptone-agar plates; reaction P_H 7.0–7.2.

      (*a*) Prepare from the stock dilution the following dilutions: 1:1,000, 1:10,000, 1:100,000.

      (*b*) With 1 c.c. of the dilutions 1:10,000 and 1:100,000 prepare pour-plates.

      (*c*) Count plates after 24 hours' aerobic incubation at 37° C.

   (3) Lactose agar plates (sugar-free-veal-infusion peptone—1 per cent lactose agar).

      (*a*) Prepare pour-plates using 1 c.c. of the dilutions 1:10,000 and 1:100,000.

      (*b*) Count plates after 48 hours anaerobic incubation at 37° C.

   (4) Spore plate:

      (*a*) Heat in a Pasteur pipette a portion of the stock dilution at 80° C. for 10 minutes.

      (*b*) Make a pour-plate of lactose agar using one-half of the heated suspension. Save the remainder for inoculation of a fermentation tube.

      (*c*) Incubate aerobically for 48 hours at 37° C.

4. Seeding of the Fermentation Tubes:

(1) 0.5 c.c. lots of the stock dilution are each seeded into the following tubes and incubated aerobically at 37° C. for 24 hours.

(*a*) 1 per cent glucose sugar-free-veal-peptone broth fermentation tube.

(*b*) 1 per cent lactose sugar-free-veal-peptone broth fermentation tube.

(*c*) 1 per cent saccharose sugar-free-veal-peptone broth fermentation tube.

(*d*) Bromcresol-purple-milk-fermentation tube.*

(2) Heated material from procedure 3, part 4, is inoculated into a fermentation tube of milk to which sterile defibrinated blood has been previously added.

(*a*) Incubate anaerobically at 37° C. for 48 hours.

5. Inoculation with Undiluted Stool:

(1) One loopful of the undiluted fecal specimen is inoculated into the following mediums:

(*a*) Gelatin stab tube; melting point 28° C.

(*b*) Loeffler's serum slant medium.

(*c*) 1 per cent lactose-peptone-ox-bile.

(2) Incubate (*b*) and (*c*) aerobically at 37° C. from 24 to 48 hours.

(3) Incubate (*a*) aerobically at 22° C. from 24 to 48 hours.

6. Acetic Acid Glucose Broth Tubes:

(1) 0.5 c.c. of the "stock dilution" is inoculated in the following tubes:

(*a*) N/5 acetic acid veal-infusion—1 per cent glucose-peptone-broth tubes.

(*b*) N/10 acetic acid veal-infusion—1 per cent glucose-peptone-broth tubes.

(c) N/20 acetic acid veal-infusion—1 per cent glucose-peptone-broth tubes.

* Clark and Lubs, *Jour. Agr. Res.*, 1917, 10, pp. 105–111.

For anaerobic cultures the plates are placed in a Novy or museum jar, from which the air is exhausted and the vacuum replaced by hydrogen. The results noted in the various culture mediums are recorded over a time interval of from 24 to 72 hours; the growth in the fermentation tubes is expressed in percentage of gas and by symbols designating the degree of turbidity. Liquefaction and digestion of gelatin and Loeffler's serum are tabulated as follows:

+ slight; ++ moderate; +++ rapid and heavy growth.

Hull, T. G., and Rettger, L. F. (*Jour. Bacteriol.*, 1917, 2, p. 47), using white rats, have made extensive studies of this nature.

1. What factors must be taken into consideration to bring about a complete aciduric flora?

2. Will simple milk feeding, unaided, transform a proteolytic flora?

3. Where is lactose digested in the intestinal tract? What conditions are necessary for its digestion?

References:

Torrey, J. C. Regulation of intestinal flora of dogs through diet. *Jour. Med. Res.*, 1919, 39, p. 415.

Rettger and Cheplin. *Intestinal Flora.* Yale Univ. Press, New Haven, 1921.

Cannon, P. R. Effects of diet on intestinal flora. *Jour. Infect. Dis.*, 1921, 29, p. 369.

Kulp, W. L., and Rettger, L. F. Comparative study of Lactobacillus acidophilus and Lactobacillus bulgaricus. *Jour. Bacteriol.*, 1924, 9, p. 357.

Kopeloff, N. Clinical results obtained with Bacillus acidophilus. *Arch. Int. Med.*, 1924, 33, pp. 47, 55.

Seventh Week

EXPERIMENT 28. PRECIPITINS

Immunize 2 rabbits against against horse serum, using both intravenous and intraperitoneal methods. The intravenous injection of 2 c.c. on 3 successive days, trial bleeding 7 days after the last injection, as recommended by Gay and Fitzgerald, can be used. Fornet and Muller injected on 3 successive days, 5, 10, 15 c.c. intraperitoneally; bleeding 12 days after the last injection. This takes a little longer but often yields a very high titer antiserum. A single animal may be immunized by a combination of both methods as follows:

Days	*Dose*	*Mode of Injection*
1	1–2 c.c.	Intravenously
7	2–4 c.c.	Intravenously
15	4–8 c.c.	Intraperitoneally
22	Trial bleeding from ear.	

In titrating immune serum, set up one or both of the following test series: Make six antigen dilutions (horse serum) 1:100, 1:500, 1:1000, 1:2000, 1:5000, and 1:10,000. After making these dilutions the antiserum titer may be determined as follows: Place 0.1 c.c. immune serum in each of six agglutination tubes, add 1 c.c. of the respective antigen dilution, shake the mixture, and observe the highest dilution in which a precipitate settles to the bottom of the tube after incubation for half an hour at 37° C.; or (2) place 1 c.c. of a 1:10 dilution of immune serum in 6 agglutination tubes, carefully stratify the antiserum with 1 c.c. of the respective antigen dilution and observe after incubation for half an hour. The end point of the titration is the highest dilution showing a white precipitin line of demarcation between the two sera.

1. Compare different methods of obtaining potent and specific precipitating sera.
2. Discuss the differentiation of animal species by means of precipitin tests.

References:

Fornet and Muller. Zur Herstellung und Verwendung praezipitierender Sera, inbesondere für den Nachweis von Pferdefleish. *Ztschr. f. Biol. Technik u. Methodik*, 1908, 1, p. 201.

Welsh, D. A., and Chapman, H. G. Differentiaion of proteins of closely related species. *Jour. Hyg.*, 1910, 10, p. 177.

GAY, F. P., AND FITZGERALD, J. G. Improved method of producing precipitins and hemolysins. *U. C. Publications in Path.*, 1912, 2 p. 77.

HEKTOEN, L. On the production of precipitins. *Jour. Infect. Dis.*, 1914, 14, p. 403.

SMITH, W. V. Methods of producing potent sera. *Jour. Med. Res.*, 1916, 34, p. 169.

FORSTER, G. F. Comparative study of precipitinogen and precipitin curves. *Jour. Infect. Dis.*, 1923, 32, p. 105.

OPIE, E. L. The relation of antigen to antibody (precipitin). *Jour. Immunol.*, 1923, 8, pp. 19, 55.

EXPERIMENT 29. PRECIPITINS

Same as preceding experiment, using watery extracts of guinea pig or horse red corpuscles. Test the immune serum with unknown cells.

1. Discuss the medico-legal application of this method of protein identification.
2. Name another equally delicate method used for protein differentiation.

References:

NUTTALL, G. H. F. *Blood Immunity and Blood Relationship.* (*Precipitin.*) Cambridge University Press, 1904.

HEKTOEN, L. Test of human blood precipitins. *Jour. Am. Med. Assoc.*, 1918, 70, p. 1273.

HEKTOEN AND SCHULHOF. Hemoglobin precipitins. *Jour. Infect. Dis.*, 1922, 31, p. 32.

EXPERIMENT 30. COCTOPRECIPITINS

Immunize 2 rabbits with heated protein solutions which have been treated in the following manner: (1) Dilute 50 c.c. sterile beef serum with 50 c.c. of sterile saline and heat at 70° C. for 30 minutes. (2) Use similar amounts as in (1) but boil for 3 minutes. Method of immunization and precipitin reaction technique are given in Experiment 28. Test the coctoprecipitins thus obtained for comparative titer, with native sera and the antigens obtained by the two methods of heating.

1. Discuss the relative sensitivity of antisera prepared by different types of antigen.
2. Does boiled milk react with a lactoserum? Which component induces the precipitin formation?

References:

SCHMIDT, W. A. Ueber ein Präzipitin, welches es ermöglicht, auch gekochtes Eiweis zu differenzieren. *Ztschr. f. Immunitätsforschung*, 1912, 13, p. 166.

ZINSSER, H., AND OTTENBERG, R. Observations on precipitin formation by boiled proteins. *Proc. N. Y. Path. Soc.*, 1914, 13, p. 78.

EXPERIMENT 31. THERMOPRECIPITIN REACTION IN ANTHRAX

Serum from a donkey immunized with anthrax culture, attenuated by heat, will be supplied. The organisms to be tested are boiled and the clear filtrate stratified with the serum. Consult the various publications on the subject, and set up the test accordingly. The unknown infection of a decomposed guinea pig or mouse cadaver should be diagnosed by means of this reaction. As controls use boiled cultures of *B. subtilis* and *B. anthracis.*

1. Why is the reaction of Ascoli of great practical value?

References:

REINHARDT. *Monatsh. f. prakt. Tierhl.,* 1920, 31, p. 268.
ASCOLI, A. *Thermoprazipitinreaktion.* Wien, Joseph Lafar, 1922, p. 7.

EXPERIMENT 32. ACTION OF SHIGA TOXIN

Two normal rabbits are given injections of *B. dysenteriae,* Shiga type, No. 159, in the following manner:

No. 1.—¼ of a 24-hour slant agar culture, subcutaneously.

No. 2.—$\frac{1}{10}$ of a 24-hour slant agar culture, intravenously. These animals are carefully autopsied when they die (3–5 days). Tissues, spinal cord included, are preserved in the usual manner for histological examination. Produce a potent toxin by growing the organism in Witte's peptone meat infusion broth P_H 6.8 for 5–7 days. Demonstrate the presence of both exo- and endotoxin by filtering the culture. Inject 1 c.c. intravenously into a young rabbit. Try anaerobic cultivation for the production of pure endotoxin. Heat the endotoxin to 70° C. for 1 hour and inject 5 c.c. intravenously into a young rabbit. *Remember this material is highly infectious!* The student should take entire charge of the animal. The animal cages should be thoroughly sterilized at the conclusion of the experiment.

1. Do your results coincide with those of Olitsky and Kliger?
2. What type of paralysis usually develops in the rabbits inoculated with Shiga toxin?

References:

GUGGISBERG. Über die Veränderungen im Zentralnervensystem bei experimenteller Dysenteriervergiftung der Kaninchen. *Arbeiten a. d. Inst. z. Erforschung d. Infektionskr.,* Bern, 1908, Heft 2.
OLITSKY, P. K., AND KLIGLER, I. J. Toxins and antitoxins of Bacillus dysenteriae Shiga. *Jour. Exper. Med.,* 1920, 31, p. 19.
MCCARTNEY, J. E., AND OLITSKY, P. K. Separation of the toxins of Bacillus dysenteriae Shiga. *Jour. Exper. Med.,* 1923, 37, p. 767.

Eighth Week

EXPERIMENT 33. AGGLUTININS IN MILK

Apply to the instructor for a culture of *B. abortus*, normal colostrum, and immune serum or colostrum. Two antigens are obtained by cultivation on 5 per cent glycerin peptic digest agar and blood agar. The antigen is washed off with saline containing 0.5 per cent formalin and placed on ice for 5 days. After testing the bacterial suspension for sterility, it is standardized by the opacity method to contain 3 billion organisms per c.c. Clear the milk with rennet. (Ref.: Smith, T., Orcutt, M. L., and Little, R. B. The source of agglutinins in the milk of cows. *Jour. Exper. Med.*, 1923, 37, p. 153.) Use the Modified Dreyer Agglutination Technic (p. 123) running the dilution series as high as 1:1000.

1. Discuss the hereditary transmission of immunity.
2. What biologic relationship exists between *B. abortus* and *B. melitensis?*
3. How may local udder infections be determined?

References:

Evans, A. C. Further studies on bacterium abortus and related bacteria. *Jour. Infect. Dis.*, 1918, 22, p. 576.

Smith, T., and Little, R. B. Cow serum as a substitute for colostrum in new-born calves. *Jour. Exper. Med.*, 1922, 36, p. 453.

Meyer, K. F., Shaw, E. B., and Fleischner, E. C. Pathogenicity of B. melitensis and B. abortus for guinea pigs. *Jour. Infect. Dis.*, 1922, 31, p. 159.

EXPERIMENT 34. LEUCOCYTOSIS

Inject a rabbit intravenously with 1 c.c. of a killed culture of Streptococcus, and follow hourly the leucocyte count until the stages of leucopenia and hyperleucocytosis have been reached and passed, respectively. Consult the theoretical studies referred to by Petersen, *Protein Therapy and Nonspecific Resistance* (New York, Macmillan Co., 1922), p. 95.

1. How are the leucocyte and temperature curves related?
2. What is the present therapeutic status of anti-streptococcus immune serum?
3. Review briefly drug therapy of septicemia.

References:

Gay, F. P., and Claypole, E. J. Specific and extreme hyperleukocytosis following the injection of Bacillus typhosus in immunized rabbits. *Jour. Am. Med. Assoc.*, 1913, 60, p. 1950.

Ozaki, Y. Spleen as a bacterial filter. *Jour. Med. Res.*, 1917, 36, p. 413; *ibid;* 1917, 37, p. 247.

Nagao, K.; Fate of India ink injected into the blood. *Jour. Infect. Dis.*, 1920, 37, p. 527.

Lund, C. C., Shaw, L. A., and Drinker, C. K. Quantitative distribution of particulate matter. *Jour. Exper. Med.*, 1921, 33, p. 231.

Fenn, W. O. Phagocytosis of solid particles. *Jour. Gen. Physiol.*, 1923, 5, p. 311.

EXPERIMENT 35. ACTION OF LEUCOCIDIN FROM STAPHYLOCOCCUS AUREUS ON LEUCOCYTES

Prepare leucocytic suspension by injecting 10 c.c. of aleuronat suspension or even plain broth in the peritoneal cavity of guinea pigs. An aleuronat suspension is best made by adding to meat extract broth 2 per cent starch and 4 per cent powdered aleuronat. This is thoroughly mixed, boiled for 5 minutes, and the smooth paste sterilized in the autoclave. A leucocidin extract is prepared by injecting 10 c.c. of broth culture into the pleural cavity of the rabbit and transferring the exudate into citrated Locke's solution 18 to 24 hours later. Determine the titer of the leucocidin, using the bioscopic method of Neisser and Wechsberg as modified by Nakayama. Use liquid petrolatum to cover the mixture of leucocytes and leucocidin.

1. Can leucocidin be employed as an antigen?

2. Is alcoholic methylene blue permissible in this reaction? Why?

3. How does temperature affect the leucocidin reaction?

References:

Neisser, M., and Wechsberg, F. Über das Staphylotoxin. *Ztschr. f. Hyg.*, 1901, 36, p. 299.

Zinsser, H., and Tsen, E. Hyperleucocytosis and its bearing on specific therapy. *Jour. Immunol.*, 1917, 2, p. 247.

Nakayama, Y. On the toxin for leukocytes produced by streptococci. *Jour. Infect. Dis.*, 1920, 27, p. 86.

EXPERIMENT 36. THE ACTION OF MACROPHAGES

Secure blood from the wing vein of a pigeon. To prevent the blood from clotting in the syringe (due to tissue thromboplastin), pour a little sterile liquid petrolatum on the skin after it has been sterilized. In order to prevent coagulation, mix a few drops of a

saturated sterile solution of sodium citrate or oxalate in the centrifuge tube in which the cells are to be washed. After washing 3 times with saline, make a 5 per cent suspension of the cells. Inject 3 c.c. of this suspension intraperitoneally into a guinea pig. The cavity may be 'prepared' by injecting 5 c.c. of sterile broth 12 hours, and again 1 hour, before introducing the foreign material. Withdraw some of the peritoneal exudate by means of a capillary pipette at 1, 3, 6, 12, 24, and 48-hour intervals after injection. Examine the material in hanging drop and stain with Wright's or Giemsa stain.

1. Which cells have been phagocytized?

2. Does any portion of the pigeon cell seem to resist digestion?

3. What is the origin and function of this type of body cell?

References:

METCHNIKOFF, E. *Immunity in Infective Diseases.* English translation by Binnie, New York, Macmillan Company, 1905.

ADDISON AND THORINGTON. Behavior of phagocytic cells of peritoneal fluid toward particulate matter. *Anat. Rec.*, 1918, 14, p. 467.

SIMPSON, M. E. Experimental production of macrophages in the circulating blood. *Jour. Med. Res.*, 1922, 43, p. 77.

MAXIMOW, A. A. Tuberculosis of mammalian tissue in vitro. *Jour. Infect. Dis.*, 1924, 34, p. 549.

GAY, F. P. Local resistance and local immunity to bacteria. *Physiol. Rev.*, 1924, 4, p. 191.

EXPERIMENT 37. THE ACTION OF MICROPHAGES ON BACTERIA

Prepare the peritoneal cavity of a guinea pig as in the last experiment. Inoculate on the following day ⅓ of a 24-hour culture of *B. dysenteriae* (Flexner). Obtain smears and stain as in last experiment at 3, 5, 10, 20, 40, 60, and 90 minute intervals.

1. What type of cells are normally found in the peritoneum?

2. Outline the stages of cell reaction and subsequent phagocytosis?

References for preceding experiment and the following:

BARTLETT, C. J., AND OZAKI, Y. Phagocytosis in vivo under various conditions. *Jour. Med. Res.*, 1917–18, 37, p. 139.

NAGAO, K. The fate of killed nonhemolytic streptococci injected into the blood and resulting cellular changes. *Jour. Infect. Dis.*, 1920, 27, pp. 327, 527.

MOTOHASHI, S. Fixed tissue phagocytes. *Jour. Med. Res.*, 1922, 43, p. 419.

FENN, W. O. Phagocytosis of solid particles. *Jour. Gen. Physiol.*, 1923, 5, p. 311.

EXPERIMENT 38. RETICULO-ENDOTHELIAL SYSTEM IN RELATION TO ANTIBODY FORMATION

Inject 2 rabbits to the saturation point with trypan blue. Then give smaller doses and attempt to prepare antisheep hemolysin or typhoid agglutinin by the Bull method (see experiments for sixth week). Always use 2 rabbits of the same weight as controls.

1. How do such experimental results affect the older theories of immunity?
2. Does trypan blue saturation affect the production of all types of immune sera in the same manner?

References given for the problem on Pyemia, page 230, and the following:

STANDENATH, F. Untersuchungen über die Bildungstätte der Präzipitine. *Ztschr. f. Immunitätsforsch. u. exper. Therap.*, 1923, 38, p. 19.

FRIEDBERGER, E. (*and associates*). Studien ueber Antikörperbildung nach Entfernung des Antigendepots. *Ztschr. f. Immunitätsforsch. u. exper. Therap.*, 1924, 39, pp. 452, 459, 462.

GAY, F. P., AND CLARK, A. R. Reticulo-endothelial system in relation to antibody formation. *Jour. Am. Med. Assoc.*, 1924, 83, p. 1296.

ASCHOFF, L. *Lectures on Pathology.* Paul B. Hoeber, New York, 1924, p. 1.

EXPERIMENT 39. OPSONINS

Immunize 2 rabbits or guinea pigs with staphylococcus. Emulsify the growth of a 24-hour old agar slant culture in 10 c.c. of saline and 'attenuate' the suspension at 56° C. for 30 minutes. The injections of the antigen should be as follows:

Day	*Amount*	
	Rabbit (intravenous injection)	*Guinea pig*
1	0.05 slant attenuated organisms	1 loopful, sbc.
2	0.05 slant attenuated organisms	
3	0.05 slant attenuated organisms	1 loopful, i.p.
4	0.05 slant attenuated organisms	
5	0.10 slant attenuated organisms	2 loopfuls, i.p.
9	0.20 slant attenuated organisms	3 loopfuls, i.p.
14	0.05 slant living organisms	6 loopfuls, i.p. (attenuated)
18	0.10 slant living organisms	1 agar slant, o.p. (attenuated)
23	0.2 slant living organisms	bleed on 24th day

A leucocytic suspension is best obtained by intraperitoneal injection of a guinea pig with 10 c.c. of sterile broth. After 12 hours the same dose is repeated. The animal is sacrificed an hour after the last

injection and the peritoneal exudate is collected aseptically. The leucocytes are washed twice with saline and a heavy suspension is finally made for the tests. Wash off the growth of a 24-hour-old agar slant culture of staphylococcus with 10 c.c. of saline. Break up any clumps of organisms by shaking the emulsion in a sterile flask with beads. Make 1:50 and 1:100 dilutions of normal and immune rabbit serum. Divide each serum dilution into 2 portions and heat one of each at 56° C. for 30 minutes.

The mixing of the respective reagents is conducted with the aid of a capillary Pasteur pipette mounted with a rubber bulb. First, the leucocytic suspension is drawn up to the mark on the pipette. A bubble of air is allowed to intervene between it and an equal volume of the bacterial suspension. This emulsion is followed in like manner by an equal amount of the serum to be tested. The 3 reagents are mixed by alternate expulsion and aspiration onto a sterile slide. The mixture is then drawn into the pipette, the tip sealed in the flame and incubated for half an hour at 37° C. After a subsequent mixing, thin films are made and fixed in alcohol-ether mixture. Stain for 2 minutes either with carbol-thionin, toluidine blue, or Giemsa stain. For quantitative comparisons the number of bacteria ingested by 100 leucocytes in each separate suspension are counted and the opsonic index determined according to the following formula:

$$\text{Opsonic index} = \frac{\text{Average no. bacteria per phagocyte—Patient's or Immune sera}}{\text{Average no. bacteria per phagocyte—Normal sera}}$$

1. How would you determine whether the active principle of the serum unites with the bacteria or with the leucocytes?

2. What is the effect of heat on opsonins?

3. State the qualifications of a satisfactory opsonic preparation.

4. How does virulence of the organism affect the rate of opsonic reaction?

References:

Manwaring, W. H., and Coe, H. C. Endothelial opsonins. *Jour. Immunol.*, 1916, 1, p. 401.

Tunnicliff, R. Specific nature of hemolytic streptococcus of scarlet fever. *Jour. Am. Med. Assoc.*, 1920, 74, p. 1386.

Cross, H. B. Modification of Wright opsonic index technic based on differential white blood count. *Johns Hopkins Hosp. Bull.*, 1922, 33, p. 142.

EXPERIMENT 40. HEMOPSONIN

Obtain fresh normal rabbit serum and also a serum from a rabbit immunized against sheep cells. Heat a portion of each serum to 56° C. for half an hour. Prepare a suspension of guinea pig or rabbit leucocytes as in the preceding experiment. Wash sheep erythrocytes 3 times and make a 5 per cent suspension. Using the latter suspension instead of bacteria, follow the technic of the preceding experiment. Incubate the mixtures for an hour and treat smears with Wright's blood-stain.

1. Which leucocytes gave the best reaction?
2. Can you explain why hemolysis has occurred in one instance?
3. Does the immune serum act upon the leucocytes or on the erythrocytes?
4. Is the hemopsonin specific for sheep cells?

Reference:

HEKTOEN, L. Specificity of opsonins in normal serum. *Jour. Infect. Dis.*, 1908, 5, p. 249.

EXPERIMENT 41. BACTERIOTROPINS

Determine the tropin titer of a sample of polyvalent antimeningococcus serum. Use the method of Evans.

1. In what respects are bacteriotropins and opsonins similar?
2. What is the therapeutic significance of tropin standardization of antisera?

Reference:

EVANS, A. C. Tropin reactions of antimeningococcus serum. *U. S. Hygienic Laboratory Bulletin*, No. 124, 1920.

Ninth Week

EXPERIMENT 42. TUBERCULOSIS INFECTION IN RABBITS

Test 2 rabbits with old tuberculin, then infect one with antiforminized sputum of a case of human tuberculosis and the other with antiforminized pus or tissue material from bovine tuberculous lesions. Control the weight and general appearance. After 5 weeks the student conducting skin tests will inject the animal with 0.1 c.c. of Koch's O. T. to see if the infection has been successful. Control animals kept in the same cages are simultaneously injected. Autopsy before the class and preserve tissues for sections.

1. What does your problem show with reference to species immunity?
2. What is the immunological significance of the lymph node lesions?
3. Trace the path of the infective material which you injected, explaining the final lesions.

References:

Foot, N. C. Endothelial reactions, endothelium in experimental general miliary tuberculosis in rabbits. *Jour. Exper. Med.*, 1921, 33, p. 271.

Haythorn, S. R. Some factors concerned in the dissemination of tubercular material in the experimental animal. *Am. Rev. Tuberc.*, 1923, 6, p. 731.

Calmette, A. Tubercle bacillus infection and tuberculosis in man and animals. Baltimore, Williams and Wilkins, 1923.

Maximow, A. A. Tuberculosis of mammalian tissue in vitro. *Jour. Infect. Dis.*, 1924, 34, p. 549.

EXPERIMENT 43. TUBERCULOSIS INFECTION IN GUINEA PIGS AND FOWLS

Antiforminize a specimen of sputum and a piece of tuberculous liver from a chicken. Inject 8 guinea pigs previously tested with tuberculin as follows: (1) Inject 2 with the straight sputum, subcutaneously, and intraperitoneally, respectively. (2) Two more are injected with the antiforminized sputum as in (1). (3) Scarify the skin of a fifth with a scalpel or sandpaper and rub into the scarifications a few drops of the antiforminized sputum with the aid of a Pasteur pipette. (4) Inject 2 guinea pigs with liver emulsion. (5) Use the eighth guinea pig as a control in the cage. Two fowls are injected intraperitoneally with antiforminized sputum and 2 with liver emulsion. Control the weight and the symptoms. The students in

charge of problem 44 will test the animals at regular intervals with human and avian tuberculin to determine the progress of the infection.

1. Can lupus be readily produced experimentally?
2. At autopsy can the place of the original injection be ascertained?
3. What is the function of the omentum?

References (See also Experiment 42):

Ryder, C. T., Olcott, C. T., and Webb, G. B. Lymph node distribution of experimental tuberculosis in the guinea pig. *Am. Rev. Tuberc.*, 1922–23, 6, p. 575.

Van Es, L., and Martin, H. M. An inquiry into the cause of the increase of tuberculosis of swine. University of Nebraska Agricultural Station, *Res. Bull.* No. 30, 1925.

EXPERIMENT 44. SKIN TEST FOR TUBERCULOSIS

Use the animals infected in experiments 42 and 43 about 4 weeks after injection. Give $\frac{1}{10}$ c.c. of a 1:10 dilution of human and avian tuberculin intracutaneously, using tuberculin syringes and sharp needles. Use some of the preparations secured in Experiment 45. Observe the reaction during the next 48 hours. For demonstration give 1 pig, which has previously shown a positive reaction, 1 c.c. of undiluted O. T. intraperitoneally; do likewise to an uninfected control. Discuss the skin test as to technic, symptoms, and significance, from a clinical standpoint.

1. State the reasons for using a special type of syringe.
2. What significance is attached to the extent of the skin reaction?
3. Can avian tuberculosis be detected by tuberculin tests?

Reference:

Calmette, A. Tubercle bacillus infection and tuberculosis in man and animals. Baltimore, Williams and Wilkins, 1923.

EXPERIMENT 45. PRODUCTION AND FRACTIONATION OF TUBERCULIN

Cultivate human tubercle bacilli in 6 liter flasks according to the method given by Calmette, page 75 (Exper. 44). Use the growth obtained from 4 of the flasks as a yield of dried organisms for the fractionation as done by Zinsser. Identify the proteose residue by precipitin reactions with immune sera.

1. What is the most delicate chemical test for protein? Biological test?

References:

VAUGHAN, V. *Protein Split Products.* New York, Lea and Febiger. 1913. p. 164.

ZINSSER, H. Studies on tuberculin reaction. *Jour. Exper. Med.*, 1921, 34, p. 507.

LONG, E. R. Chemical evidence on phylogenetic classification of tubercle bacillus. *Am. Rev. Tuberc.*, 1923, 8, p. 195.

EXPERIMENT 46. DISTRIBUTION OF ORGANISMS IN THE BUCCAL CAVITY

Demonstrate (1) the behavior of charcoal in the buccal cavity when placed on the tongue and (2) the distribution of *B. prodigiosus* in the mouth by the methods outlined by Bloomfield.

Discuss the importance of droplet infection.

References:

BLOOMFIELD, A. L. Mechanism of respiratory tract infection. *Tubercle*, 1924, 5, p. 438.

BLOOMFIELD, A. L. Mode of transmission of Streptococcus tonsilitis. *Johns Hopkins Hosp. Bull.*, 1924, 35, p. 115.

BLOOMFIELD, A. L. The mechanism of elimination of bacteria from the respiratory tract. *Am. Jour. Med. Sci.*, 1922, 164, p. 854. See also *Am. Rev. Tuberc.*, 1922, 5, p. 903.

EXPERIMENT 47. PREPARATION OF DIPHTHERIA TOXIN

Use one of the methods described in the references.

1. Enumerate the methods used to free the media of fermentable sugars.
2. What advantages are gained by 'ripening' toxin?
3. In what forms is toxin used and stored?

References:

British Medical Research Council. *Diphtheria.* His Majesty's Stationery Office, London, 1923, p. 113.

DERNBY, K. G., AND DAVID, H. Study on preparation of diphtheria toxin. *Jour. Path. and Bacteriol.*, 1921, 24, p. 150.

DERNBY, K. G., AND WALBUM, L. E. Studien über die Bildung des Diphtheria toxins. *Biochem. Ztschr.*, 1923, 138, p. 505.

WALBUM, L. E. Das Diphtheria toxin. *Abderhalden's Handbuch d. biol. Arbeitsmethoden.*, 1924, Abt. XIII, part 2, p. 595.

EXPERIMENT 48. DIPHTHERIA ANATOXIN

Immunize 2 rabbits with anatoxin and determine antitoxic unitage by Römer technic or Ramon flocculation procedure.

1. In what respects is anatoxin superior to toxin for immunization?
2. Which component of the toxin is destroyed by the action of the formalin?

References:

ZINGHER, A. Modification of Roemer's intracutaneous method for determination of small amount of diphtheria antitoxin in blood sera. *Jour. Infect. Dis.*, 1916, 19, p. 557.

RAMON, G. Sur le propriétés de l'anatoxine diphtherique. *Compt. Rend. Acad. d. Sci.*, 1924, 179, p. 422.

GLENNY, A. T., HOPKINS, B. E., AND POPE, C. G. Further notes on the modification of diphtheria toxin by formaldehyde. *Jour. Path. and Bacteriol.*, 1924, 27, p. 261.

GLENNY, A. T., AND OKELL, C. C. The titration of diphtheria toxin and antitoxin by flocculation methods. *Jour. Path. and Bacteriol.*, 1924, 27, p. 187.

BAYNE-JONES, S. The titraton of diphtheria toxin and antitoxin by Ramon's flocculation method. *Jour. Immunol.*, 1924, 9, p. 481.

O'BRIEN, R. A. Active immunization against diphtheria. *Lancet*, 1925, 1, p. 102.

EXPERIMENT 49. NATURAL IMMUNITY OF RATS TO DIPHTHERIA

Technic of Kolmer, (ed. 3; W. B. Saunders Co., 1923, p. 1119), as follows:

(1) Secure diphtheria toxin in which the M. L. D. and L_+ doses are known.

(2) Inject each of 2 white rats intraperitoneally with 1000 M. L. D. Inject a third rat with 10,000 M. L. D.

(3) Next morning bleed one of the first 2 rats and inject the serum into a guinea pig (intraperitoneally).

(4) Dilute a portion of the toxin with saline solution in such manner that 0.2 c.c. carries 1/50 of the L_+ dose.

(5) Secure blood from additional normal rats and defibrinate; centrifuge and secure the serum.

(6) In a small test tube place 0.2 c.c. of the diluted toxin and 0.8 c.c. of the serum; in a second tube place 0.2 c.c. of the toxin and 0.8 c.c. of saline solution. Allow mixtures to stand 30 minutes.

(7) Secure a 10- to 12-ounce guinea pig; pluck the hairs away at 3 places on the abdomen. Inject 0.1 c.c. of each of the 3 mixtures *intracutaneously*.

(8) Observe all of the animals daily for evidences of inflammatory reactions and necrosis.

1. Is the immunity of the rat relative or absolute?
2. Is the rat naturally immune to diphtheria toxin? Is the guinea pig?
3. Is natural diphtheria antitoxin present in rat serum?
4. Upon what does the natural immunity of the rat to diphtheria depend?

Reference:

British Medical Research Council. *Diphtheria.* His Majesty's Stationery Office, London, 1923.

EXPERIMENT 50. SCHICK TEST

The members of the class must submit to the Schick test for diphtheria immunity. Follow the method outlined by Zingher (0.2 c.c. containing 1/40 M. L. D.). Place the test on the right arm and the control on the left arm. Read the reactions after 48 hours and 5 days, respectively.

Schick Test: A standard diphtheria toxin is at first diluted 1 to 10 in carbolized saline. From this a further dilution of such strength is made that 0.1 c.c. contains 1/40 M. L. D. for the guinea pig. For example:

M. L. D. 1/125 c.c.: Dilute 1 c.c. of toxin with 11.5 c.c. of carbolized saline = total dilution 12.5 c.c.; in this primary Schick dilution there are 10 M. L. D. in each c.c. To prepare the final Schick dilution, dilute 1 c.c. of G. P. D. with 39.0 c.c. of carbolized saline. 1 c.c. = ¼ M. L. D.; 0.1 c.c. = 1/40 M. L. D.

For the purpose of checking the reaction, a control toxin heated for 10 minutes at 75° C. is used. The primary dilution is made as described for the unheated toxin. The final dilution is then prepared by diluting 1.25 c.c. of the primary dilution with 39 c.c. of saline. The 0.1 carries a trifle excess of the heated toxin.

1. Which constituents of the toxic solution are responsible for the large number of pseudo reactions?
2. How can a positive reaction be determined upon a dark skinned person, i.e. Negro?
3. What is known about sex, age, and race immunity to diphtheria?

References:

SCHICK. *Munich med. Wochnschr.*, 1913, 60, p. 2608.

WEAVER, G. H., AND RAPPAPORT, B. Further observations on the Schick test for diphtheria immunity. *Jour. Am. Med. Assoc.*, 1916, 66, p. 1448.

WRIGHT, LOUIS T. The Schick test with especial reference to the Negro. *Jour. Infect. Dis.*, 1917, 21, p. 265.

ZINGHER, A. Diphtheria prevention work in the public schools of New York City. *Jour. Am. Med. Assoc.*, 1921, 77, p. 835.

ZINGHER, A. The Schick test. *Jour. Am. Med. Assoc.*, 1922, 78, p. 490.

EXPERIMENT 51. KELLOGG TEST

Test the sera of at least 10 members of the class and correlate the results with those of the Schick test. Follow the procedures outlined by Kellogg, W. H., The intracutaneous guinea pig test. *Jour. Am. Med. Assoc.*, 1923, 80, pp. 748, 1399, and *ibid.*, 1923, 81, p. 678. The students giving positive Schick reactions will be immunized by the instructor with toxin-antitoxin mixtures.

1. What advantages has this procedure over the Schick test?
2. How is the skin dose of toxin standardized?
3. Why must a control injection of toxin be placed on every guinea pig? Why is a serum control not necessary?

References:

ZINGHER, A. The intracutaneous guinea pig test for human susceptibility and immunity to diphtheria. *Jour. Am. Med. Assoc.*, 1923, 80, pp. 1023, 1867.

PARK, W. H. Human hypersensitiveness to whole horse serum or serum globulins following diphtheria toxin antitoxin injections.—Its importance. *Jour. Immunol.*, 1924, 9, p. 17.

SEARS, E. W. Observations on Schick and toxin-antitoxin immunization against diphtheria in City of Auburn. *Am. Jour. Public Health*, 1924, 14, p. 210.

EXPERIMENT 52. VIRULENCE AND TOXICITY TESTS OF DIPHTHERIA BACILLI

Compare the field culture method of Force, J. N., and Beattie, M. (*Amer. Jour. Hyg.*, 1922, 2 p. 490) and the subcutaneous method with pure cultures, as described by Kolmer (ed. 3, p. 97). For the comparison use at least 6 cultures; for the second part of the problem, purify the cultures very carefully.

Demonstrate the anatomical lesions to the class.

1. Which method is the most practical?
2. Under what circumstances are virulence tests of great value?
3. Why is a small dose of antitoxin given the test animal 5 hours after injection of the emulsion? Why does it not cause a negative test?

References:

EAGLETON, A. J., AND BAXTER, E. M. The virulence of diphtheria-like organisms. *Brit. Med. Jour.*, 1921, 1, p. 775.

HALL, J. W. Behavior of diphtheria bacilli in acidified media, especially with reference to virulence tests. *Jour. Hyg.*, 1922–23, 21, p. 155.

KELLY, F. L., AND POTTER, A. The administrative value of the virulence test for diphtheria bacilli. *Jour. Am. Med. Assoc.*, 1923, 81, p. 734.

BULL, C. C., AND MCKEE, C. M. Whole-culture method of testing the virulence of diphtheria bacilli. *Am. Jour. Hyg.*, 1923, 3, p. 103.

POWELL, H. M. A biological study of diphtheria bacillus. *Am. Jour. Hyg.*, 1923, 3, p. 109.

EXPERIMENT 53. STANDARDIZATION OF DIPHTHERIA ANTITOXIN

In order to economize animals use Römer's intracutaneous technic as described by Römer, Ueber den Nachweis sehr kleiner Mengen des Diphtheriegiftes (*Ztschr. f. Immunitätsforsch.*, 1909, 3, p. 208). In case the experiment is not conclusive, repeat the tests by the method outlined in Coca, *Essentials of Immunology*, 1925, pp. 134–136.

1. How can the toxicity of growing cultures of diphtheria bacilli be determined quickly and cheaply?
2. Is there any relationship between the L_f and the L_0 or L_+ dose?
3. Is the standard Hygienic Laboratory method more reliable than the Römer technic?

References:

RÖMER, P. H., AND SAMES, T. Zur Bestimmung sehr kleiner Mengen Diphtherieantitoxins. *Ztschr. f. Immunitätsforsch.*, 1909, 3, p. 344.

RÖMER, P. H., AND SOMOGYI, R. Eine einfache Methode der Diphtherieserumbewertung. *Ztschr. f. Immunitätsforsch.*, 1909, 3, p. 433.

ZINGHER, A. Modification of Roemer's intracutaneous method for determination of small amounts of diphtheria antitoxin in blood sera. *Jour. Infect. Dis.*, 1916, 19, p. 557.

For further information, consult:

ARRHENIUS AND MADSEN. State Serum Institute, Denmark, 1902, 3, p. 1–97.

ROSENAU, M. J. The immunity unit for standardizing diphtheria antitoxin. *Hyg. Lab. Bull. No. 21*, 1905.

RAMON, G. Flocculation dans un mélange neutre de toxine-antitoxine diphtériques. *Compt. Rend. Paris Soc. de Biol.*, 1922, 86, p. 661.

RAMON, G. Sur une technique de titrage in vitro du sérum antidiphtérique. *Compt. Rend. Paris Soc. de Biol.*, 1922, 86, pp. 711–813.

GLENNY, A. T., AND OKELL, C. C. The titration of diphtheria toxin and antitoxin by flocculation methods. *Jour. Path. and Bacteriol.*, 1924, 27, p. 187.

BAYNE-JONES, S. Titration of diphtheria toxin and antitoxin by Ramon's flocculation method. *Jour. Immunol.*, 1924, 9, p. 481.

Tenth Week

EXPERIMENT 54. EXPERIMENTAL PYEMIA

Inject 2 rabbits intravenously with the unstrained suspension scraped from a 48-hour potato slant culture of *Staphylococcus pyogenes aureus*. Several days preceding the injection, determine the temperature fluctuations, weight, and blood count of the animals to be injected. Continue the observations after the injection and record the changes in the blood picture. Make differential counts at daily intervals. Sacrifice the animals during the third or fourth week and demonstrate the lesions to the class.

1. Why is potato used for the bacterial culture?
2. What is meant by elective localization of bacteria?
3. Discuss the mechanism of tissue localization of bacteria in different animals.

References:

Koch, J. Über Beziehungen der Staphylokokken und Streptokokken zu den Gallenwegen. *Ztschr. f. Hyg. u. Infectionskrankh.*, 1908, 60, p. 335; *ibid.*, 1908–09, 62, p. 1; *ibid.*, 1911, 69, p. 436.

Ito, H. Fate of staphylococcus introduced into normal dog. *Jour. Med. Res.*, 1917, 37, p. 189.

Ozaki, Y. Spleen as a bacterial filter—Phagocytosis of bacteria. *Jour. Med. Res.*, 1917, 36, p. 413; 37, p. 247.

Nagao, K. Fate of India ink injected into the blood. *Jour. Infect. Dis.*, 1920, 27, p. 527.

Lund, C. C., Shaw, L. A., and Drinker, C. K. Quantitative distribution of particulate material (Manganese dioxide) administered intravenously to the dog, rabbit, guinea pig, rat, chicken and turtle. *Jour. Exper. Med.*, 1921, 33, p. 231.

Fenn, W. O. Phagocytosis of solid particles. *Jour. Gen. Physiol.*, 1923, 5, p. 311.

EXPERIMENT 55. AGGLUTINATION OF STAPHYLOCOCCI

Immunize at least 3 rabbits with different chromogenic strains of staphylococci. By means of such antisera, classify a number of other strains supplied by the instructor, and study the cross-agglutination reactions. Use the methods described in the references.

1. Do virulent strains fall into definite serological groups?
2. Is color production correlated serologically?

References:

JULIANELLE, L. A. Studies of hemolytic staphylococci. *Jour. Infect. Dis.*, 1922, 31, p. 256.

HINE, T. G. M. Serological classification of staphylococci. *Lancet*, 1922, 2, p. 1380.

SEEDORFF, JOHAN. Staphylococci—Serological and Fermentative Classification of Types. Levin and Munksgaard, Copenhagen, 1923.

EXPERIMENT 56. STAPHLOLYSIN

Inoculate 2 flasks of beef extract peptone bouillon with a virulent strain of *Staphylococcus aureus*. Add sufficient sterile glucose solution to one flask to make a 1 per cent sugar media. Titrate hemolysin in each flask daily with 1 per cent suspension human, sheep, and rabbit cells. Test culture for the presence of a lipolytic ferment. (See Orcutt and Howe.) Six test tubes are necessary for each species of cells used. Place 1 c.c. of cell suspension in each tube and add 0.1, 0.2, 0.4, 0.8, 1.0, and 2 c.c. of the centrifugalized supernatant fluid from the culture. Add sufficient saline to make a final 3.0 c.c. volume in each tube. A saline — cell suspension control and a cell — sterile bouillon control are necessary. Incubate 2 hours and read the degree of hemolysis. Doubtful tubes around the end point of hemolysis should be cleared by 5 minutes centrifugalization before reading.

1. Is the hemolytic activity lost by cultivation on plain agar?
2. To what biological factor is the lysis due?
3. Does the hemolysin production follow the growth curve of the organism?

References:

WALBUM, L. E. Studien über die Bildung der Bakterien Toxin Staphylosin. *Biochem. Ztschr.*, 1922, 129 p. 367.

ORCUTT, M. L., AND HOWE, P. E. Hemolytic action of a staphylococcus due to a fat splitting enzyme. *Jour. Exper. Med.*, 1922, 35, p. 409.

JULIANELLE, L. A. Studies of hemolytic staphylococci, hemolytic activity—biochemical reactions, serologic reactions. *Jour. Infect. Dis.*, 1922, 31, p. 256.

EXPERIMENT 57. EXOTOXIN OF CERTAIN STRAINS OF STAPHYLOCOCCUS AUREUS

Follow the method and technic described by Parker, J. T., Production of exotoxin by certain strains of Staphylococcus aureus. (*Jour. Exper. Med.*, 1924, 40, p. 761.)

EXPERIMENT 58. ANTISTAPHYLOLYSIN

Follow directions as given by Kolmer (ed. 3, p. 1127), as follows:

(1) Prepare a staphylolysin by growing a culture of *Staphylococcus aureus* in bouillon for 2 or 3 days. Pass through a Berkefeld filter and preserve the filtrate with 0.5 per cent phenol. Determine the lytic dose for 1 c.c. of a 1 per cent suspension of rabbit corpuscles.

(2) Secure serum from a rabbit immunized with staphylococci. Heat at 56° C. for 30 minutes.

(3) Secure normal horse serum. Heat at 56° C. for 30 minutes.

(4) Secure normal rabbit serum. Heat at 56° C. for 30 minutes.

(5) Into a series of 6 small test tubes place the lytic dose of staphylolysin and increasing amounts of rabbit immune serum as follows: 0.001, 0.005, 0.01. 0.05, 0.1, 0.2 c.c. Arrange a similar series, using normal horse serum and normal rabbit serum. Prepare a control containing the lytic dose of toxin. Add 1 c.c. of a 1 per cent suspension of rabbit cells to each tube and sufficient normal salt solution to make the total volume equal 2 c.c. Shake each tube gently and incubate at 37° C. for 2 hours.

(6) Inspect the tubes. The smallest amount of normal horse serum inhibiting hemolysis is taken as 1, or the unit. Compare the values of the immune and normal rabbit serums with this unit.

1. What is the nature of the antistaphylolysin? Is it an antiferment?
2. Explain the mechanism of lysin-antilysin action.
3. Which rôle does the lysin play in staphylococcus infections?
4. Of what value would be the titration of antistaphylolysin in the serum of the patient?

EXPERIMENT 59. SEPTICEMIA

Inoculate a rabbit intravenously with a broth culture of a pathogenic strain of *Streptococcus pyogenes*. Make blood cultures daily during life; cultures and sections from the organs after death. Make leucocyte counts at 2-hour intervals for the first 10 hours.

1. How are the leucocyte and temperature curves related?
2. What is the present therapeutic status of anti-streptococcus immune serum?
3. Review briefly drug therapy of septicemia.

References:

ALLISON, C. S. Bactericidal action of arsenical compounds on experimentally produced streptococcic septicemias. *Jour. Med. Res.*, 1918, 38, p. 55.

GAY, F. P., AND STONE, R. L. Experimental Streptococcus empyema, attempts at prevention and therapy by means of vaccines and serum. *Jour. Infect. Dis.*, 1920, 26, p. 265.

GAY, F. P., AND MORRISON, L. F. Experimental Streptococcus empyema. Attempts at dye therapy. *Jour. Infect. Dis.*, 1921, 28, p. 1.

YOUNG, H. H., AND HILL, J. H. Treatment of septicemia and local infections by intravenous injections of mercurochrome and gentian violet. *Jour. Am. Med. Assoc.*, 1924, 82, p. 669.

EXPERIMENT 60. EMPYEMA

Inject into the pleural cavity of a rabbit the last generation of the passage strain of *Streptococcus pyogenes* used by Gay and Stone. Remove and examine daily some of the fibrino-purulent exudate.

1. What organisms usually cause pleurisy in man? Under what conditions?

2. Would this passage strain of streptococcus have localized in the pleura if injected intravenously?

References:

GAY, F. P., AND STONE, R. L. Experimental Streptococcus empyema. Attempts at prevention and therapy by means of vaccines and serum. *Jour. Infect. Dis.*, 1920, 26, p. 265.

KARSNER, H. T., AND SWANBECK, C. E. Removal of particulate matter from the pleura. *Jour. Med. Res.*, 1920, 42, p. 91.

GAY, F. P., AND CLARK, A. R. Clasmatocytes and passive immunity to streptococcus infections. *Jour. Infect. Dis.*, 1925, 35, p. 233.

EXPERIMENT 61. STREPTOCOCCUS HEMOLYSIN

Use a 10 to 24-hour culture of a virulent strain of streptococci grown in serum infusion broth P_H 7.8. Test the effect of the addition of glucose to the medium upon hemolysin production. Titer hemolytic property with 1 per cent suspension of guinea pig, human, rabbit, and sheep cells. Set up a series of 6 tubes for each species of cells used. Place 1 c.c. of cell suspension in each tube and add 0.1, 0.2, 0.4, 0.8, 1.0, and 2 c.c. of the centrifugalized supernatant fluid from the culture. Controls of saline-cell suspension and sterile bouillon-cells should be set up. Add sufficient saline to make a final volume of 3.0 c.c. Incubate for 2 hours and read the degree of hemolysis. Test culture for lipase. Determine the temperature at which the active principle is destroyed.

1. What is the difference between a hemolysin and a hemotoxin?
2. Why is it unnecessary to add guinea pig complement to cause hemolysis?
3. Does the hemolysin curve parallel the growth curve?

References:

Meader, P. D., and Robinson, G. H. Biological and physical properties of the hemotoxin of streptococci. *Jour. Exper. Med.*, 1920, 32, p. 639.

de Kruif, P. H., and Ireland, P. M. Streptolysin. *Jour. Infect. Dis.*, 1920, 26, p. 285.

Cook, M. W., Mix, V., and Culvyhouse, E. O. Hemotoxin production by the streptococcus in relation to its metabolism. *Jour. Infect. Dis.*, 1921, 28, p. 93.

Mishulow, L. Acute respiratory infections. Differences in the character of the hemolytic action of streptococci and the relative value of various methods in demonstrating these differences. *Jour. Immunol.*, 1921, 6, p. 329.

EXPERIMENT 62. EFFECT OF SURFACE TENSION ON GROWTH OF STREPTOCOCCI

Use either the Du Noüy or drop method of measuring surface tension of various media at different hydrogen-ion concentrations.

1. What is the effect of lowered surface tension upon pellicle formation?
2. Does any correlation exist between the usual biological classification of streptococci and the groups established through surface tension measurements?

References:

Du Noüy, P. L. New apparatus for measuring surface tension. *Jour. Gen. Physiol.*, 1919, 1, p. 521.

Larson, W. P., Cantwell, W. F., and Hartzell, T. B. Influence of surface tension of culture media on growth of bacteria. *Jour. Infect. Dis.*, 1919, 25, p. 41.

Larson, W. P. Surface tension of culture media. *Abstr. Bacteriol.*, 1921, 5, p. 2.

Ayers, S. H., Rupp, P., and Johnson, W. T. Influence of surface tension. Depressants on the growth of streptococci. *Jour. Infect. Dis.*, 1923, 33, p. 202.

EXPERIMENT 63. AGGLUTINATION OF STREPTOCOCCI

Produce at least 2 different streptococcus-agglutinating sera with organisms from scarlet or erysipelas cases. Choose your own method of immunization. If necessary, concentrate the agglutinins by the method of Tunnicliff and Herrold. (See experiment 66.) Consider carefully the physical factors affecting the agglutination of streptococci as outlined by Shibley. Study the group relation of a number of other strains of streptococcus isolated from various sources.

1. Summarize the main difficulties of the early workers on this problem.

2. What electrolyte gives the best agglutination flocculation with this type of microörganisms?

References:

Dochez, A. R., Avery, O. T., and Lancefield, R. C. Antigenic relationships between strains of Streptococcus hemolyticus. *Jour. Exper. Med.*, 1919, 30, p. 179.

Tunnicliff, R. Studies on the specificity of streptococci. *Jour. Am. Med. Assoc.*, 1920, 75, p. 1339.

Valentine, E., and Mishulow, L. Cultural and serological relationships of hemolytic streptococcus from inflammatory conditions of respiratory tract. Jour. Immunol., 1921, 6, pp. 301, 329, 343.

Gordon, M. H. Serological differentiation of Streptococcus pyogenes from Streptococcus scarletinae. *Brit. Med. Jour.*, 1921, 1, p. 632.

Bliss, W. P. Antigenic relationships between strains of Streptococcus hemolyticus isolated from scarlet fever. *Jour. Exper. Med.*, 1922, 36, p. 575.

Norton, J. F. Serologic relationships in Streptococcus viridans group. *Jour. Infect. Dis.*, 1923, 32, p. 37.

Shibley, G. S. Agglutination of streptococci. *Jour. Exper. Med.*, 1924, 39, p. 245.

Stevens, F. A., and Dochez, A. R. Agglutination and absorption agglutins with Streptococcus scarletinae. *Jour. Exper. Med.*, 1924, 40, p. 253.

EXPERIMENT 64. FOCAL INFECTION IN THE RABBIT

Secure from the instructor a pair of excised human tonsils. Sterilize the surface by dipping in hot paraffin oil (180° C. for 5 seconds). By means of sterile instruments, cut through the tonsils and cut several small pieces from different regions of the freshly cut surface. Macerate the small pieces in a mortar or sterile petri dish. Inoculate a small amount onto the surface of blood agar plates and the remainder into deep glucose brain broth tubes according to the

method of Rosenow. After 18–24 hours' growth, shake tube, examine the growth microscopically, make surface and dilution plates. Inoculate 5 c.c. of the 18–24-hour brain broth culture (after centrifugalization at low speed for a few minutes to remove pieces of brain) intravenously into 4 young rabbits. Observe the animals carefully; sacrifice on the third to fifth day. Autopsy carefully (including joints), and make complete cultures in deep glucose brain broth tubes.

References:

Davis, D. J. Bacteriology and pathology of the tonsils. *Jour. Infect. Dis.*, 1912, 10, p. 148.

Rosenow, E. C. The newer bacteriology of various infections as determined by special methods. *Jour. Am. Med. Assoc.*, 1914, 63, p. 903.

Rosenow, E. C. The bacteriology of cholecystitis and its production by injection of streptococci. *Jour. Am. Med. Assoc.*, 1914, 63, p. 1835.

Rosenow, E. C. Elective localization of streptococci. *Jour. Am. Med. Assoc.*, 1915, 65, p. 1687.

Billings, F.: *Focal Infection.* The Lane Medical Lectures on Focal Infection. New York, D. Appleton and Co., 1916.

McMeans, J. W. Experimental bacteremia. *Arch. Int. Med.*, 1918, 22, p. 617.

MacNevin and Vaughan. *Abstract of the Literature on Mouth Infections and Their Relation to Systemic Disease.* New York, Joseph Purcell Research Memorial, 1922, Parts I and II.

Eleventh Week

EXPERIMENT 65. DICK TEST FOR SCARLET FEVER SUSCEPTIBILITY

The entire class will submit to the test. The following procedure is recommended: The stock toxin is diluted with carbolized saline (usually 1:1000); one-half of diluted toxin is held in a water bath at 100° C. for 1 hour. The technic of injection is the same as in the Schick test: 0.1 c.c. of diluted toxin is injected intradermally on the right forearm for the test and a similar amount of heated toxin on the left forearm for the control. The reading of the reaction must begin about 12–16 hours after injection.

1. In what respects is *Streptococcus scarlatinae* toxin peculiar compared to other types of toxin?
2. Does the skin reaction last as long as in a Schick test? Is there pigmentation?
3. How do you account for the negative tests on people who have no history of scarlet fever?

References:

Mulsow, F. W. Blanching of the skin by serum injection in scarlet fever. *Jour. Infect. Dis.*, 1921, 29, p. 557.

Zingher, A. The Dick test in normal persons and in acute and convalescent cases of scarlet fever. *Jour. Am. Med. Assoc.*, 1924, 83, p. 432.

Dick, G. H., and Dick, G. F. A skin test for susceptibility to scarlet fever. *Jour. Am. Med. Assoc.*, 1924, 82, p. 265.

Dick, G. H., and Dick, G. F. Scarlet fever toxin in preventative immunization. *Jour. Am. Med. Assoc.*, 1924, 82, p. 544; *ibid.*, 1925, 84, p. 1477.

Okell and Parrish. The Dick test in scarlet fever. *Lancet*, 1925, 1, p. 712.

EXPERIMENT 66. DEMONSTRATION OF AGGLUTININS IN THE SERUM OF CONVALESCENT SCARLET FEVER PATIENTS

Prepare *Streptococcus scarlatinae* antigen. (See Experiment 63.) Concentrate the immune bodies in the serum of convalescent scarlet fever cases, which will be supplied, by the method of Tunnicliff, R., and Herrold, R. D., Specific streptococcus agglutinins in concentrated scarlatinal serum (*Jour. Infect. Dis.*, 1924, 34, p. 209).

1. Is this the best method for diagnostic purposes? Why?
2. How is wound scarlet usually recognized?

EXPERIMENT 67. PRODUCTION OF STREPTOCOCCUS SCARLATINAE TOXIN

Use the method outlined by Dick and Dick., The scarlet fever toxin and the Dick test. *Jour. Am. Med. Assoc.*, 1925, 84, p. 1477.

1. What constitutes a good toxin?

References:

Dick, G. H., and Dick, G. F. Scarlet fever toxin in preventative immunization. *Jour. Am. Med. Assoc.*, 1924, 82, p. 544.

Williams, Banghof and Hussey. *Proc. Soc. Exper. Biol. and Med.*, 1924, 21, p. 291.

Huntoon, F. M. Properties of the Dick scarlatinae toxin. *Proc. Soc. Exper. Biol. and Med.*, 1924, 21, p. 2487.

EXPERIMENT 68. PROTECTIVE VALUE AND SPECIFICITY OF ANTIPNEUMOCOCCUS SERUM TYPE I

Conduct the tests on mice by the method described in Monograph 7, Rockefeller Institute for Medical Research, 1917, pp. 56–60. Use normal horse serum and Type III serum as controls.

1. What practical application is made of such protective tests?
2. State the precautions which must be observed when horses are to be used in the production of antisera for therapeutic use.
3. How effective are polyvalent antisera therapeutically?

References:

Thomas, W. S. Type I. Pneumonia and its serum treatment. *Jour. Am. Med. Assoc.*, 1921, 77, p. 2101.

Locke, E. A. Treatment of type I. Pneumococcus lobar pneumonia with specific serum. *Jour. Am. Med. Assoc.*, 1923, 80, p. 1507.

EXPERIMENT 69. EXPERIMENTAL LOBAR PNEUMONIA IN THE RABBIT

Etherize a rabbit. Introduce a soft rubber catheter into the trachea, passing it well down into the bronchus. Inject 5 c.c. of a 60-hour glucose serum broth culture of virulent pneumococcus. Follow by a syringeful of air to force the culture into the terminal bronchi. Follow the temperature and leucocytes. When the animal dies, autopsy carefully and take cultures. Embed tissues for histological studies.

1. Are the temperature and leucocyte curves comparable to those of a human case of pneumonia?
2. Are there any evidences of pleuritis? If so, how do you explain the infection of the pleura?

References:

WINTERNITZ, M. C., AND HIRSCHFELDER, A. D. Studies on experimental pneumonia in rabbits. *Jour. Exper. Med.*, 1913, 17, p. 657.

BLAKE, F. G., AND CECIL, R. L. Production of pneumococcus. Lobar pneumonia in monkeys. *Jour. Exper. Med.*, 1920, 31, p. 403.

EXPERIMENT 70. ISO-AGGLUTININS AND ISO-HEMOLYSINS

(1) Collect 10–20 drops of blood from each member of the class in 5 c.c. of saline 1 per cent sodium citrate. Centrifuge and wash cells once with normal saline. Make a 1 per cent cell suspension. Obtain sera of Groups II and III from instructors. Use the open slide method of typing with a large drop of both sera and cell suspension; rotate gently, not longer than 15 minutes.

(2) Bleed at least 10 members of the class from the arm vein, taking 3–5 c.c. for serum and 1 c.c. for 1 per cent cell suspension. Begin this work when the class is collecting serum (sixth week) for Widals to avoid extra bleedings. Test serum against known type cells and, vice versa, as in a blood transfusion for donner and recipient by the macroscopic test. Use 0.5 c.c. serum and 0.5 c.c. cell suspension and incubate. Control cell suspension for hemolysis. Use Jansky's classification as follows:

Cells of Groups	Serum of Groups			
	I	II	III	IV
I	—	—	—	—
II	+	—	+	—
III	+	+	—	—
IV	+	+	+	—

Serum of Groups	Cells of Groups			
	I	II	III	IV
I	—	+	+	+
II	—	—	+	+
III	—	+	—	+
IV	—	—	—	—

1. Can hemolysis be seen by the slide method? Why?
2. Does hemolysis ever occur without agglutination?
3. What other kinds of fluids besides blood are given intravenously in large quantities for critical illness? Under what conditions?

References:

Read first the various references, consult Coca: *Essentials of Immunology.* Baltimore, Williams and Wilkins Co., 1925, p. 137.

Landsteiner, K. Zur Kenntniss der antifermentativen lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe. *Centralbl. f. Bakteriol.,* 1900, 27, p. 357.

Moss. Studies in iso-agglutinins and iso-hemolysins. Technic. *Johns Hopkins Hosp. Bull.,* 1910, 21, p. 63.

Hirshfeld, L., and Hirshfeld, H. Blood grouping on Macedonian frontier. *Lancet,* 1919, 2, p. 675.

Sanford. Blood transfusion. *Med. Clin. N. Amer.,* 1919, 3, p. 801.

Lewis, J. H., and Henderson, D. L. The racial distribution of iso-hemagglutining groups. *Jour. Amer. Med. Assoc.,* 1922, 79, p. 1422.

Ottenberg, R. Medicolegal application of human blood grouping. *Jour. Am. Med. Assoc.,* 1922, 79, p. 2137.

Buchanan, J. A. Medicolegal application of the blood group. *Jour. Am. Med. Assoc.,* 1922, 78, pp. 78, 89.

Guthrie, C. G., Pessel, J. F., and Huck, J. G. Further studies on blood grouping. *Johns Hopkins Hosp. Bull.,* 1924, 35, p. 221.

EXPERIMENT 71. MUTUAL MATCHING OF STUDENTS' SERA AND CORPUSCLES

According to Coca, *Essentials of Immunology* (Baltimore, Williams and Wilkins, 1925), p. 145.

Materials Required—

10 slides and an equal number of cover slips for each student.
Capillary pipettes with rubber nipples; serological pipettes.
White paper, wax pencils, wooden applicators.
Saline and 10 per cent sodium citrate solution.

Procedure (conduct the work during the sixth week when blood for Widals is being collected from each student): Defibrinate about 2–3 c.c. of blood with the wooden applicators. After centrifugalizing, the students transfer the supernatant serum (being careful not to disturb the corpuscular sediment) into another serological tube. One-half cubic centimeter of the corpuscular sediment is transferred to a third serological tube and thoroughly mixed with 2 c.c. of physiological saline solution.

Each student is given a number for purposes of identification, and, in turn, passes from desk to desk, depositing on the slide, at the end bearing his number, one drop of his own suspended blood corpuscles. Each student thus receiving the drop of suspended corpuscles mixes with it a drop of his own serum and covers the mixture with a cover slip, which should be moved slightly from time to time with needle or wooden applicator. In this way finally, the serum of each student is tested with the blood corpuscles of all of the other students.

The results of the mutual tests are charted and it can be seen which students possess inagglutinable corpuscles (Group I individuals); which possess non-applutinating serum (Group IV individuals);

and which are divided into 2 groups that show cross-agglutination. These two groups are Nos. II and III. As a rule No. II is the larger group.

EXPERIMENT 72. CLINICAL MATCHING OF BLOODS

According to COCA, A. F., *Essentials of Immunology* (Baltimore, Williams and Wilkins, 1925), p. 140. The students are paired for carrying out the direct matching of bloods.

"A simple method of performing the direct matching of two bloods is the following, devised by Coca and based upon suggestions by James Ewing, Levine and Mabee, and L. B. Drake.

The stem of a white blood-cell-counting pipette is filled with 1 per cent sodium citrate solution up to the 0.5 mark. The finger of the recipient is punctured as for a blood count, and blood is drawn into the pipette until the upper level of the citrate solution reaches the 1 mark. The blood and citrate solution are expelled upon a spot near 1 end of a glass slide, and mixed with the end of the pipette. Care should be taken to avoid the formation of air bubbles. After the pipette has been rinsed with 1 per cent sodium citrate, and again filled up to the 0.5 mark with that solution, an equal volume of the donor's blood is drawn into the pipette and deposited with the citrate solution near the end of the slide. Two divisions of this thoroughly mixed donor's blood are mixed with the whole of the citrated recipient's blood. This mixture is taken up in the stem of the pipette, and, after being deposited on the center of another glass slide, it is covered with a clean $\frac{7}{8}$ inch square cover slip. The cover slip is moved slightly from time to time, until agglutination is observed, or until 15 minutes have elapsed. On account of the preponderance of recipient's corpuscles in the mixture, the observation of the clumping must be conducted with the aid of a microscope. Rouleaux formation can be distinguished from true agglutination with the use of the high power dry lens. The individual corpuscles can be distinguished in groups of rouleaux, but not in true agglutination.

The use of the citrate solution serves a double purpose: it prevents coagulation of the specimens and it provides a saline dilution of the blood mixtures, which, in some instances, is necessary to hasten the agglutination, so that this reaction will take place within the 15 minutes prescribed for the examination. In other words, instances have been encountered in which agglutination failed to occur after as long as one half-hour, when the agglutinating serum became mixed with agglutinable corpuscles that had not been washed or diluted with saline solution."

Reference:

OTTENBERG, R. A Classification of human races based on geographic distribution of blood groups. *Jour. Am. Med. Assoc.*, 1925, 84, p. 1393.

Twelfth Week

General References dealing with hemolysin phenomena:

Gay, F. P., and Fitzgerald, J. G. An improved rapid method of producing precipitins and hemolysins. *U. C. Publ. Path.*, 1912, 2, p. 77.

Coca, Arthur F. A rapid and efficient method of producing hemolytic amboceptor. *Jour. Infect. Dis.*, 1915, 17, p. 361.

Morrison, L. F. On the origin and nature of alexin (complement) in guinea pig blood. *Jour. Immunol.*, 1922, 7, p. 435.

Stowell, F. R., and Beattie, M. I. Comparison of methods for production of anti-sheep hemolysin. *Am. Jour. Hyg.*, 1925, 5, p. 1.

The hemolysin unit will be determined as in the *Standard Wasserman Technic*, method 3 (Kolmer, ed. 3; Philadelphia, W. B. Saunders, 1923, p. 481), as follows:

Titration of Hemolysin

Tube	Hemolysin	Complement 1:30	Corpuscles, 2 per cent		After Water-bath Incubation for one hour
1	0.5 cc. 1: 1,000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
2	0.5 cc. 1: 2,000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
3	0.5 cc. 1: 3,000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
4	0.5 cc. 1: 4,000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
5	0.5 cc. 1: 5,000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis.
6	0.5 cc. 1: 6,000	0.3 cc.	0.5 cc.	1.7 cc.	Complete hemolysis; unit.
7	0.5 cc. 1: 8,000	0.3 cc.	0.5 cc.	1.7 cc.	Marked hemolysis.
8	0.5 cc. 1:10,000	0.3 cc.	0.5 cc.	1.7 cc.	Marked hemolysis.
9	0.5 cc. 1:12,000	0.3 cc.	0.5 cc.	1.7 cc.	Slight hemolysis.
10	0.5 cc. 1:16,000	0.3 cc.	0.5 cc.	1.7 cc.	No hemolysis.

EXPERIMENT 73. NORMAL HEMOLYSINS OF DOG OR GOAT

Technic of Zinsser *et al.* (ed. 2; New York, Macmillan Co., 1921), p. 22, as follows:

The normal blood serum of many animals has haemolytic and haemagglutinative action upon the red blood cells of animals of some other species. This haemolytic action, like the bactericidal action, is lost when the serum is heated to 56° C. The haemagglutinating property, on the other hand, is relatively resistant to heat and is not destroyed until the serum is heated to 70° C. or more. Such a haemolytic normal serum acts only on the erythrocytes of certain other species, sometimes upon 2 or 3 varieties with differing intensity. Thus goat serum is strongly haemolytic for rabbit cells—less so for those of guinea pigs. Dog serum contains a relatively large amount of normal haemolysin for most cells used in laboratory work. Guinea pig cells are highly susceptible, and human cells relatively resistant to haemolysis by dog serum.

REAGENTS: Fresh dog serum, 2 per cent suspension of washed guinea pig cells and human cells, also saline. Place 0.25 c.c. of dog serum in a test tube and heat for one-half hour in water bath at 56° C. Bring up volume to 2.5 c.c. with normal salt solution. Prepare a similar 1–10 dilution of unheated dog serum. Set up the following experiment in half-inch tubes:

(1)	Dog serum unheated	(1–10)	0.5 c.c. + Guinea pig cells 2 per cent	0.5 c.c.
(2)	Dog serum unheated	(1–10)	0.5 c.c. + Human cells 2 per cent	0.5 c.c.
(3)	Dog serum heated	(1–10)	0.5 c.c. + Guinea pig cells 2 per cent	0.5 c.c.
(4)	Dog serum heated	(1–10)	0.5 c.c. + Human cells 2 per cent	0.5 c.c.
(5)	Salt solution		0.5 c.c. + Guinea pig cells 2 per cent	0.5 c.c.
(6)	Salt solution		0.5 c.c. + Human cells 2 per cent	0.5 c.c.

Incubate the tubes 1 hour at 37° C. in water bath, observing changes in cells at intervals of 5, 10, 15, 30, and 60 minutes. Tabulate the results. Tube 1 should show prompt haemolysis, tube 3 should show agglutination of the cells. In tubes 2 and 4 there should be no observable change in the cells in most samples of dog serum.

1. Under what circumstances must the hemolysin relation of species be determined?

Reference:

KAHN, R. L., AND LYON, D. S. Complement fixation—On the affinity of sheep corpuscles for antisheep hemolysin. *Jour. Infect. Dis.*, 1921, 29, p. 651.

EXPERIMENT 74. PROPERTIES AND TITRATION OF COMPLEMENT

Bleed 6 guinea pigs asceptically from the heart for serum. Make 2 samples of a 1:20 dilution of the serum. Titrate daily against 2 units of sheep amboceptor (Wasserman Standard). Prepare a graph of the rate of deterioration of the diluted and undiluted serum held at room and ice-box temperatures. Filter a portion of the 1:20 serum through a Berkefeld candle and ascertain if any of the active principle has been absorbed.

Make 1:300 saline dilutions of normal sodium hydroxide and hydrochloric acid. To 1 c.c. of 1:10 sera add an equal amount of each chemical dilution and place in a 37° C. water bath for half an hour. What is the effect of such treatment upon the titer of the complement? The activity of the complement derived from the serum of animals of various species should be studied. Take 0.1 c.c. amounts of complement and amboceptor; dry it at room temperature upon separate pieces of filter paper of appropriate size. The following day and 2 weeks later titrate the strength of the complement and amboceptor. Dissolve the sera on the filter paper by soaking it in a measured amount of saline before setting up the hemolytic series.

1. What animal sera is the most potent in complement fixation tests?
2. How is complement best preserved?
3. What relation do the results of treatment with acid and alkali bear upon the handling of glassware for complement fixation tests?

References:

MORRISON, L. E. Origin and nature of complement. *Jour. Immunol.*, 1922, 7, p. 435.

COCA, A. F. *Essentials of Immunology.* Baltimore, Williams and Wilkins, 1925, pp. 165, 168.

EXPERIMENT 75. ARTIFICIAL HEMOLYSIS

Immunize 3 rabbits with sheep or goat erythrocytes by several methods mentioned by Stowell and Beattie, A Comparison of Methods for the Production of Anti-sheep Hemolysins in Order to Determine the Day on which the Higest Titer is Obtained (*Amer. Jour. Hyg.*, 1925, 5, p. 1).

1. How do your observations coincide with theirs?
2. Does complement unite with the corpuscles?
3. How may cells be sensitized?

EXPERIMENT 76. HETEROPHYLIC HEMOLYSINS

Immunize 2 rabbits with extracts of beef heart or guinea pig renal cells and test the hemolytic properties of the rabbit sera against sheep, guinea pig, dog, and human erythrocytes.

1. What bearing has this phenomena on the doctrine of specificity?
2. May other types of material besides proteins produce hemolysins of this variety?
3. Are the cells of all species equally susceptible to heterologous hemolysins?

References:

TANIGUCHI, T. Studies on heterophile antigen and antibody. *Jour. Path. and Bacteriol.*, 1922, 25, p. 77.

IIJIMA, T. Presence of heterophile antigen in bacteria. *Jour. Path. and Bacteriol.*, 1923, 26, p. 519.

VON FRIEDE, K. A. New heterogentic antigen and antibody of guinea pigs. *Klin. Wochnschr.*, 1924, 3, p. 1226; *idem., Abstr. Jour. Am. Med. Assoc.*, 1924, 83, p. 482.

POWELL, H. M. Immunization with heterophile antigen when given by mouth. *Am. Jour. Hyg.*, 1925, 5, p. 228.

EXPERIMENT 77. EFFECT OF HEMOLYTIC SERA IN VIVO

Two rabbits should be immunized with either cat or guinea pig cells. If cats are to be used, test their blood sera for complement; use a sheep cell sensitizer system. The technic given by Zinsser *et al.* (ed. 2; New York, Macmillan Co., 1921), p. 84, is as follows:

A rabbit receives at 4-day intervals 4 intravenous injections of 2 c.c. of washed, cat's red blood cells. For obtaining the cells a cat is bled under ether from the heart. The defibrinated cat's blood, if sterile, can be kept 4 days and thus used for 2 injections. The rabbit is bled 7 to 10 days after the last injection and the hemolytic and agglutinative power of the inactivated immune serum for cat's cells is tested.

Etherize 2 cats and inject 1 c.c. of immune serum into one and 3 c.c. into the other. Determine colormetrically the percentage of hemoglobin in the blood before injection and at various times afterward. Give the injections directly into the jugular vein or the saphenous vein of the hind leg with the aid of a fine hypodermic needle. Put the first cat into a clean cage with a drain for the collection of urine after 6, 24, and 48 hours. (The first urine passed will contain large amounts of hemoglobin, the last, bile pigments.) Half an hour after the injection collect a specimen of the cat's serum by pricking the ear vein. Centrifuge and observe the blood-tinged serum. Make smears of the blood, stain by Wright's method, and note with the oil-immersion lens the phagocyted red cells in some of the leucocytes. The second cat will probably die at once. If so, autopsy immediately and look for clumps of agglutinated red cells in portal and pulmonary veins. Examine smears from the spleen and liver for phagocytized cells.

To demonstrate the *in vivo* effect of complement, give a rabbit an intravenous injection of 2.0 c.c. of 25 per cent suspension of sensitized sheep blood cells. (In sensitizing the cells, regard 0.1 c.c. of a 25 per cent cell suspension as a unit and add 2 units of amboceptor for every unit of cells. After the cells have stood in contact with the amboceptor for one-half hour, centrifugalize them and make them up to the original volume of 25 per cent suspension.)

After half an hour collect a specimen of serum from the rabbit's ear and examine for dissolved hemoglobin. Make a smear of the blood and examine for phagocytosis. Examine the rabbit's urine after 6, 24, and 48 hours.

1. How is the presence of hemoglobin in urine determined chemically?

2. What percentage of erythrocytes must be destroyed before hemoglobin appears in the urine?

3. Does a hemolytic antiserum cause cell agglutination above the hemolytic zone?

References:

CARREL, A., AND EBELING, A. H. Leucocytic secretions. *Jour. Exper. Med.*, 1922, 36, p. 645.

PHENOMENON OF COMPLEMENT FIXATION

General Technic. KOLMER (ed. 3; Philadelphia, W. B. Sounders Co., 1923), p. 1144, as follows:

(1) Dilute 1 c.c. of guinea pig serum complement with 9 c.c. of saline solution (1:10) and titrate in doses of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 c.c. against 2 units of hemolysin and 1 c.c. of 2½ per cent sheep corpuscle suspension, or use any other reliable hemolytic system.

(2) Secure 1 c.c. of an antibacterial and a normal serum and heat each at 56° C. for 30 minutes.

(3) Secure suspension or extract of young culture of the organism to be studied. This is called the antigen; for the proper dose to use, consult the instructor.

(4) Proceed as follows:

Tube 1: 0.2 c.c. antiserum + dose of antigen + 2 units of complement + normal salt solution.

Tube 2: 0.2 c.c. antiserum + 2 units of complement + normal salt solution (serum control).

Tube 3: 0.2 c.c. normal serum + dose of antigen + 2 units of complement + normal salt solution.

Tube 4: 0.2 c.c. normal serum + 2 units of complement + normal salt solution (serum control).

Tube 5: dose of antigen + 2 units of complement + normal salt solution (antigen control).

Tube 6: 2 units of complement + normal salt solution (hemolytic control).

Tube 7:1 c.c. corpuscle suspension + normal salt solution (corpuscle control).

(5) Shake all tubes gently and incubate for 1 hour at 37° C.

(6) Add 2 units hemolysin and 1 c.c. corpuscle suspension to all tubes except corpuscle control. Shake gently and reincubate for 1 hour.

1. Examine tubes and record your results.
2. Why was the serum control on each serum necessary?
3. Why was the hemolytic system control necessary?
4. Why was the antigen control necessary?
5. What is meant by non-specific complement fixation?
6. What is meant by specific complement fixation? Explain the phenomenon. Which tube of this series shows specific complement fixation, and why? Is there any evidence of non-specific fixation in any of the tubes? If so, what bearing would this have on the results of this test?
7. What is meant by complement deviation? By complement fixation?
8. Upon what does the specificity of complement fixation depend?

EXPERIMENT 78. BORDET-GENGOU REACTION FOR B. TYPHOSUS PREPARATION AND TITRATION OF ANTIGEN

Use the method of Kolmer (ed. 3, Philadelphia, W. B. Saunders Co., 1923), p. 1149, given below; the ordinary agglutinating sera can be used.

(1) Cultivate typhoid bacilli upon slants of agar for 48 to 72 hours.

(2) Wash these off with sufficient saline solution to give a cloudy suspension. Shake with glass beads for 1 hour and heat at 60° C. for 1 hour. Add 0.05 c.c. of a 5 per cent solution of phenol for each cubic centimeter of antigen.

(3) Titrate the antigen for anticomplementary activity and use it in dose corresponding to ¼ this amount.

(4) Conduct complement-fixation tests with the human sera if sera from typhoid fever patients are available. If not, employ rabbit typhoid immune serum heated to 60°–62° C. for 30 minutes. If the author's new technic is employed the maximum dose should be not more than 0.025 c.c., to avoid non-specific reactions.

1. Describe different methods for the preparation of bacterial antigens.
2. In what diseases of human beings and the lower animals are complement-fixation tests of practical diagnostic value?
3. Why does complement fixation fail in some bacterial diseases?
4. Why is complement fixation generally stronger in syphilis than in other bacterial diseases?
5. Why is a sensitive technic especially desirable in bacterial complement-fixation tests?

Reference:

BORDET, J., AND GAY, F. P. *Studies in Immunity.* New York, John Wiley and Sons, 1909.

EXPERIMENT 79. BORDET–GENGOU REACTION FOR C. DIPHTHERIAE

Titrate the sensitizer in the immune serum prepared on 2 rabbits by the inoculation of a toxic strain of *C. diphtheriae.* Consult preceding experiment. Use Kolmer complement-fixation technic.

References:

STONE, R. L. Fixation reactions with types of Diphtheriae. *Jour. Infect. Dis.*, 1924, 34, p. 312.

DOYLE, D. Further study of agglutination of Bacillus diphtheriae. *Jour. Immunol.*, 1924, 9, p. 443.

EXPERIMENT 80. ANTICOMPLEMENTARY PROPERTY OF EGG ALBUMIN AND OF ANTI-EGG-WHITE SERUM

NON-SPECIFIC AND SPECIFIC COMPLEMENT FIXATION

Use method outlined by Coca, A. F., *Essentials of Immunology* (Baltimore, Williams and Wilkins, 1925), p. 168. Begin to immunize 4 rabbits with egg white. Space the injections as follows: first day: 1 c.c. of a sterile 50 per cent egg white solution intravenously. Fifth day: 10 c.c. of a sterile 50 per cent egg white solution intraperitoneally. Tenth to fifteenth day: test the serum by the 'ring' method for precipitin (p. 214).

1. Are protein amboceptors specific?
2. Does the complement-fixing power of a serum depend upon its precipitin titer?
3. How does serum precipitation affect the hemolytic zone?

EXPERIMENT 81. DIAGNOSTIC COMPLEMENT FIXATION FOR TUBERCULOSIS

Use the complement fixation technic of Kolmer, with an antigen of tubercle bacilli and sera from the following sources:

(1) Individuals with acute pulmonary tuberculosis.

(2) Individuals with chronic fibroid tuberculosis.

(3) Healthy person with negative v. Pirquet tuberculin reaction.

(4) Syphilitic person with negative v. Pirquet tuberculin reaction.

(5) Sera of guinea pigs and rabbits with typical lesions.

1. Is tuberculosis complement fixation sufficiently specific to be reliable diagnostically? Under what conditions?

2. Does syphilitic infection of a patient vitiate the results? Why?

Reference:

WADSWORTH, A., MALTANER, F., AND MALTANER, E. Study of the complement fixation reaction in tuberculosis. *Jour. Immunol.*, 1925, 10, p. 241.

Thirteenth Week

General references on hypersensitiveness:

Novy, F. G., and de Kruif, P. H. Anaphylatoxin and anaphylaxis. *Jour. Am. Med. Assoc.*, 1917, 68, p. 1524. (Series of articles in *Jour. Infect. Dis.*, 1917.)

Wells, H. G. *Chemical Pathology.* (Ed. 4; Philadelphia, W. B. Saunders, 1920), p. 193.

Coca, A. F. Hypersensitiveness. *In* Tice, *Practice of Medicine* (1920), p. 107.

Wells, H. G. Present status of problems of anaphylaxis. *Physiol. Rev.*, 1921, 1, p. 44.

Wells, H. G. *Chemical Aspects of Immunity* (New York, Chemical Catalog Co., 1925), p. 196.

EXPERIMENT 82. ACTIVE ANAPHYLAXIS IN GUINEA PIGS

Guinea pigs (*a*) and (*b*) receive 0.2 c.c. horse serum diluted with 1.8 c.c. salt solution intraperitoneally.

Guinea pigs (*c*) and (*d*) receive 0.2 c.c. sheep serum diluted with 1.8 c.c. salt solution intraperitoneally.

Three weeks after the sensitizing injection the pigs are again injected intraperitoneally as follows:

(*a*) 1 c.c. of horse serum.

(*b*) 1 c.c. of sheep serum.

(*c*) 1 c.c. sheep serum.

The results in (*a*) and (*c*) demonstrate anaphylactic shock.

The absence of symptoms in (*b*) demonstrate specificity.

Guinea pig (*d*) is given 0.01 c.c. sheep serum subcutaneously 4 times, at 1 hour intervals. Observe carefully symptoms after each injection. Then give 1.0 c.c. sheep serum intraperitoneally. Symptoms will be slighter than in (*a*) and (*c*). This demonstrates desensitization.

1. What anatomic changes occur in an acute fatal anaphylactic shock?
2. How does the mode of injection affect the result?
3. What relation exists between agglutination or precipitin titer and the period of anaphylactic sensitivity?

References:

Parker, W. H. Importance of human hypersensitiveness following diphtheria toxin-antitoxin injections. *Jour. Immunol.*, 1924, 9, p. 17.

Hooker, S. B. Human hypersensitiveness induced by very small amounts of horse serum. *Jour. Immunol.*, 1924, 9, p. 7.

EXPERIMENT 83. PASSIVE ANAPHYLAXIS PRODUCED BY RABBIT ANTIHORSE SERA

Zinsser *et al.* (ed. 2; New York, Macmillan Co., 1921), p. 184, as follows:

Obtain serum of a rabbit which had been highly immunized to horse serum. Make preliminary serum titration of antihorse precipitin.

Inject 1.0 c.c. of this serum intravenously into each of 3 guinea pigs. After 24 hours inject into the heart as follows:

Guinea pig (*a*) 0.1 c.c. horse serum.

Guinea pig (*b*) 0.3 c.c. horse serum.

Guinea pig (*c*) 1.0 c.c. horse serum.

Observe symptoms.

In case of anaphylactic death, immediately open chest and observe condition of lungs and also whether heart is still beating. While such an animal is dying it is important to watch carefully the gradual alteration of respirations, the great irregularity and slowing of breathing, and the intense inspiratory efforts just before death.

1. Would these guinea pigs be sensitive to rabbit or horse sera if they live?
2. Is the reaction humoral or cellular?
3. Does sensitization among animal species vary?

References:

Gay, F. P., and Scuthard, E. E. On serum anaphylaxis in the guinea-pig. *Jour. Med. Res.*, 1907, 16, p. 143.

Bordet, J. Present views on anaphylaxis. *Johns Hopkins Hosp. Bull.*, 1921, 32, p. 269.

EXPERIMENT 84. ANAPHYLACTIC SHOCK IN THE RABBIT

Use the rabbits which have received egg white injections. Follow the procedures outlined by Coca, *Essentials of Immunology* (Baltimore, Williams and Wilkins, 1925), p. 160. Demonstrate the presence of precipitin in the serum.

The demonstrator injects about 5 c.c. of a 50 per cent solution of egg white intravenously into the rabbit having a 1:4000 precipitin titer. This rabbit should die within 5 to 10 minutes with the symptoms of acute anaphylactic shock in this animal. Immediately after the death of the animal, the chest is opened, and attention is called by the demonstrator to the collapsed condition of the lungs. The lower ⅔ portion of the 2 ventricles is cut off with scissors, and a large glass cannula is firmly ligated in the pulmonary artery. The pinchcock on the rubber tubing connecting the cannula with the glass bulb is released, and the bulb is raised slowly until the tubing is fully

extended in a perpendicular position, or until fluid begins to ooze from the left ventricle. The degree of pressure of the column of saline solution can be observed in the ballooning of the pulmonary artery about the end of the cannula. While this pressure is still being maintained, the tip of one of the lungs may be cut off with the scissors, and attention may be called to the fact that no fluid escapes from the cut capillaries or arterioles. Further portions may be cut off until an artery is cut behind the zone of constriction, whereupon the fluid issues in a sudden stream.

The demonstrator may now dissect out the trachea, insert a pipette in the upper end, and, grasping the cut lobe tightly, he can show that the remainder of the lung is easily inflated and deflated.

Reference:

Coca, A. F. Mechanism of anaphylaxis reaction in rabbit. *Jour. Immunol.*, 1919, 4, p. 219.

EXPERIMENT 85. ANAPHYLAXIS IN VITRO

Inject 2 female guinea pigs (200 gms.) with egg white and 1 with horse dander or any other atopen. Test 12–16 days later the hypersensitiveness of the uterus by the Schulz-Dale technic as outlined by Walzer and Grove, Studies in Specific Hypersensitiveness (*Jour. Immunol.*, 1925, 10, p. 498).

1. Does the same musculative react to anaphylactic shock in various animal species?
2. Are anaphylactic tests for medico-legal work as specific as the precipitin reaction?

References:

Dale and Laidlaw. Method standardization pituitary extracts. *Jour. Pharm. and Exper. Therapy*, 1914, 5, p. 75.

Weil, R. Studies in anaphylaxis. *Jour. Immunol.*, 1916–17, 2, pp. 95, 109, 299, 469, 525.

EXPERIMENT 86. LOCAL ANAPHYLACTIC REACTIONS THE ARTHUS PHENOMENON

Obtain 1 or 2 of the rabbits used for the preparation of precipitins, also a normal rabbit. At least 4–5 weeks should have elapsed after the last inoculation of antigen. Shave the abdomen and inject each animal with 0.1 c.c. of the sensitizing antigen, intracutaneously.

Note any reaction at the site of injection during the first couple of hours and again after 24, 48 and 72 hours.

1. Does the skin reaction parallel precipitin formation?
2. How is this phenomenon related to the humoral and cellular theories of immunity?
3. When may a negative skin test be obtained on a sensitized animal?

References:

ARTHUS, M. *Comp. Rend. Soc. de Biol.*, 1903, 55, p. 817; *ibid.*, 1906, 60, p. 1143.

WELLS, C. W. On the specific precipitin in the blood of persons injected with antidiphtheric horse serum. *Jour. Infect. Dis.*, 1915, 16, p. 63.

WEIL, R. Studies in anaphylaxis. *Jour. Immunol.*, 1916–17, 2, pp. 95, 109, 299, 469, 525.

ZINSSER, H. Studies on the tuberculin reaction and on specific hypersensitiveness in bacterial infection. *Jour. Exper. Med.*, 1921, 34, p. 495.

OPIE, E. L. Arthus phenomena. *Jour. Immunol.*, 1924, 9, pp. 231–259.

EXPERIMENT 87. DESENSITIZATION OR ANTIANAPHYLAXIS

Sensitize 2 guinea pigs with beef or horse serum (1 c.c. on first day, 2 c.c. on fifteenth day injected into the rectum). On the thirtieth day after sensitizing the guinea pig, inject 0.0001 c.c. of the anaphylactogen every 15 minutes for 1½ hours. On the sixteenth day after sensitizing, inject a guinea pig with 1 c.c. of antigen. If this animal develops anaphylactic shock, inject the other 3 guinea pigs in a similar manner.

1. Do anaphylactic symptoms develop? If not, why not?
2. What method is generally employed in the effort to induce an antianaphylactic state in serum therapy?

EXPERIMENT 88. HYPERSENSITIVENESS

Test a number of students who are sensitive to one or several atopens (pollen, epidermal substance, foods, etc.). Select the best method given in one of the references.

1. Are all the atopens proteins?
2. How do drug idiosyncrasies differ from true anaphylactic reactions?

References:

WALKER, C. I. Asthma. *Oxford Medicine*, vol. 2, p. 217.

ALEXANDER, H. L., AND PADOCK, R. Bronchial asthma, response to pilocarpin and epinephrin. *Archiv. Inter. Med.*, 1921, 27, p. 184.

SELFRIDGE, G. Disturbances of metabolism and its relation to disorders of respiratory tract. *Laryngoscope*, 1922, 32, p. 243.

RACKEMAN, F. M. Skin tests with foreign proteins. *Am. Jour. Med. Sci.*, 1922, 163, p. 87.

COCA, A. F. Specific hypersensitiveness—The preparation of fluid extracts and solutions for use in use in the diagnosis and treatment of the allergies with notes on the collection of pollens. *Jour. Immunol.*, 1922, 7, p. 163.

COOKE, R. A. Studies in specific hypersensitiveness—New etiologic factors in bronchial asthma. *Jour. Immunol.*, 1922, 7, p. 147, 219.

EXPERIMENT 89. LOCAL PASSIVE SENSITIZATION OF THE HUMAN SKIN TO ATOPENS

Method as outlined by Coca. Each student receives in one arm an intradermal injection of 0.1 c.c. of filtered serum of a human individual sensitive to one atopen (horse, rabbit, cat dander, or some pollen), and in the other arm a similar injection of the serum of an individual sensitive to some other atopen.

The next day an injection of about 0.01 to 0.02 c.c. of a solution of the respective atopen is made with a fine needle in one of the passively sensitizen sites in each student, and a similar injection of a non-related atopen is made in the other site. Both atopens are injected in the same quantity in normal sites for control. After 10 minutes, note the reactions and interpret the results.

Reference:

Coca, A. F. *Essentials of Immunology.* Baltimore, Williams and Wilkins Co., 1925.

EXPERIMENT 90. HAY FEVER AND DRUG IDIOSYNCRASIES

Select students from the class who are hypersensitive to pollens. Prove the hypersensitiveness by skin tests and outline a method of treatment.

1. What hereditary relation governs the transmission to offspring of this type of hypersensitiveness?
2. Does the genus of the plant correlate with the sensitivity of the individual?

References:

Walker, C. I. Hay fever. *Oxford Medicine,* vol. 2, p. 243; *ibid.,* 1919, pt. 1, p. 115.

Selfridge, G. Vaso-motor disturbances of nose with special reference to hay fever. *Laryngoscope,* 1920, 30, p. 611.

Coca, A. F. Specific hypersensitiveness—The preparation of fluid extracts and solutions for use in the diagnosis and treatment of the allergies with notes on the collection of pollens. *Jour. Immunol.,* 1922, 7, p. 163; *ibid.,* 1925, 10, p. 555.

Duke, W. W. Specific tests in diagnosis of allergy. *Archiv. Inter. Med.,* 1923, 32, p. 298.

Piness, G., Miller, H., and Alles, G. A. Comparative methods of protein extraction with chemical and clinical studies. *Jour. Am. Med. Assoc.,* 1924, 83, p. 608.

Peshkin, M. M. Bronchial asthma and other allergic manifestations in pharmacists. *Jour. Am. Med. Assoc.,* 1924, 82, p. 1854.

Opie, E. L. "Skin sensitivity." *Jour. Immunol.,* 1924, 9, pp. 231–259.

EXPERIMENT 91. PREPARATIONS OF FLUID EXTRACTS AND SOLUTIONS FOR USE IN THE DIAGNOSIS OF ATOPIC CONDITIONS

A number of properly prepared students will prepare wheat and house-dust atopens by the method of Coca.

References:

Wodehouse, R. P. Preparation of vegetable proteins for anaphylactic tests. *Boston Med. and Surg. Jour.*, 1916, 175, p. 195.

Clock, R. O. Comparative value of methods of preparing pollen antigens. *Jour. Infect. Dis.*, 1917, 21, pp. 387, 523.

Wodehouse, R. P., and Olmstead, J. M. D. Preparation of animal food and vegetable proteins for anaphylactic tests. *Boston Med. and Surg. Jour.*, 1917, 176–77, pp. 85, 467.

Wodehouse, R. P. Immunochemistry of proteins of dog hair, cat hair and horse dander. *Jour. Immunol.*, 1917, 2, p. 243.

Osborne, T. B., and Wells, H. G. Anaphylaxis reactions with purified proteins from milk. *Jour. Infect. Dis.*, 1921, 29, p. 200.

Lewis, Wells, Hoffman, and Gartner. Chemical and immunological study of alcohol-soluble proteins of cereals. *Proc. Soc. Exper. Biol. and Med.*, 1924, 22, p. 185.

Piness, G., and Alles, G. A. Comparative methods of protein extraction with chemical and clinical studies. *Jour. Am. Med. Assoc.*, 1924, 83, p. 608.

Parker, J.T. The production of precipitins for ragweed pollen. *Jour. Immunol.*, 1924, 9, p. 515.

New and Non-official Remedies. Chicago, American Medical Association, 1924, p. 244.

Coca, A. F., and Milford, E. L. The preparation of fluid extracts and solutions for use in the diagnosis and treatment of atopic condition. *Jour. Immunol.*, 1925, 10, p. 555.

EXPERIMENT 92. ZOOTOXIN—COBRA VENOM

Technic of Kolmer (ed. 3; Baltimore, W. B. Saunders Co., 1923, p. 1116), as follows:

(1) Collect about 2 c.c. of normal human blood and place in 5 c.c. of 2 per cent sodium citrate in 0.85 per cent sodium chloride solution. This mixture must not be shaken and the cells should be washed at least 4 times with normal salt solution, after which they are made up to a 4 per cent suspension.

(2) Prepare a 2 per cent suspension of sheep corpuscles in the same manner.

(3) Prepare a stock 1:5000 dilution of cobra venom by dissolving 0.005 gm. dried venom in 10 c.c. of normal salt solution. This solution will keep about 1 week in the refrigerator.

(4) Prepare a solution of venom 1:10,000 by diluting 2 c.c. of the stock solution with 2 c.c. salt solution. Prepare a 1:20,000 dilution by diluting 2 c.c. of dilution 1:10,000 with 2 c.c. salt solution.

(5) Place 1 c.c. of corpuscle suspension into each of 3 small test tubes and add 1 c.c. of each dilution of venom respectively. Shake the tubes gently and place in the incubator for an hour and then in the refrigerator over-night.

(6) Repeat, using rabbit and guinea pig corpuscles.

(7) Inject 2 c.c. of the 1:5000 dilution intravenously into a rabbit and 1 c.c. intravenously into a guinea pig.

(8) Observe the animals closely, particularly the urine.

1. Does the venom show a hemotoxic action? Does it act the same on rabbit and guinea pig corpuscles? If not, why not?
2. How do you explain venom hemolysis?
3. What are the evidences of venom hemolysis in vivo?
4. Is the hemotoxic poison the most dangerous constituent of venom?

References:

Coca, A. Toxins and the side-chain theory. *Jour. Infect. Dis.*, 1915, 17, p. 351.

Kyes, P. Venom hemolysis. *Jour. Infect. Dis.*, 1910, 7, p. 181.

Bulglia. Chemical analysis of snake venoms. *Chem. Abstr.*, 1924, 18, p. 1674.

Mason, C. C., and Rockwold, R. Physio-chemical aspects of hemolysis. *Jour. Lab. and Clin. Med.*, 1924, 10, p. 10.

EXPERIMENT 93. NEPHROTOXIN

Technic of Kolmer (ed. 3; Baltimore, W. B. Saunders Co., 1923, p. 1153), as follows:

(1) Prepare an emulsion of guinea pig kidney and immunize a rabbit.

(2) Inject a guinea pig intravenously with 5 c.c. of rabbit immune serum per kilo of body weight.

(3) Place the animal in a metabolism cage, collect all urine, and carefully examine for albumin, casts, and hemoglobin; make quantitative albumin determinations.

(4) After 3 days autopsy and examine the kidneys and liver histologically.

(5) Heat the immune serum to 56° C. for 30 minutes and place increasing amounts in a series of test tubes as follows: 0.01, 0.05, 0.08, 0.1, and 0.2 c.c.; add 1 c.c. of fresh guinea pig complement serum 1:20 and 1 c.c. of 2½ per cent suspension of washed guinea pig erythrocytes. Incubate for 2 hours.

1. Describe the changes occurring in the liver and kidneys. Give the prevailing views regarding the mechanism of their production.
2. Are there any evidences of a hemolytic action of the serum *in vivo? In vitro?*
3. How do you explain the presence of hemolysin in this immune serum?
4. Discuss the specificity of cytotoxins in general.
5. How may a hemolysin be removed from a nephrocytotoxic serum?
6. Have the cytotoxins any practical value in the treatment of disease?

Fourteenth Week

EXPERIMENT 94. INFLUENCE OF TEMPERATURE ON NATURAL IMMUNITY OF HEN AGAINST TETANUS

Kolmer (ed. 3; Baltimore, W. B. Saunders Co., 1923, p. 1119) as follows:

(1) Procure dried tetanus toxin and dissolve in 6 c.c. normal salt solution an amount equivalent to 6 lethal doses for a 350 gram guinea pig.

(2) Inject 2 c.c. into the pectoral muscles of a young hen. Take the rectal temperature.

(3) Inject 2 c.c. into a second hen. Take the rectal temperature and place her in cold water until the temperature has dropped several degrees.

(4) Place the first hen in a cage and keep her at ordinary laboratory temperature.

(5) Place the second hen in a cage and keep her in a cold place or renew the bath several times in order to keep her temperature subnormal.

1. What is the normal temperature of the hen?
2. Do both animals present symptoms of tetanus?
3. How do you explain the difference?

(6) If the first hen does not show symptoms of tetanus by the second day, reinject her with an amount of toxin equal to 10 lethal doses for a guinea pig.

1. Does this hen present evidences of tetanus?
2. If so, how do you explain the result?

EXPERIMENT 95. NATURE OF THE TOXIN–ANTITOXIN REACTION ACTION OF ANTITETANOLYSIN

Kolmer (ed. 3; Baltimore, W. B. Saunders Co., 1923, p. 1127) as follows:

(1) Secure fresh tetanus toxin and determine the dose producing complete hemolysis of 1 c.c. of a 1 per cent suspension of rabbit corpuscles in 2 hours at 37° C.

(2) Place double this dose of toxin in a series of 6 small test tubes and add increasing doses of fresh tetanus antitoxin: 0.001, 0.005, 0.01, 0.05, 0.1, and 0.2 c.c. Add salt solution to bring the total volume to 1 c.c.; incubate at 37° C. for 1 hour. Add 1 c.c. of 1 per cent suspension of rabbit corpuscles to each tube. Prepare 2 controls, one with the dose of toxin and corpuscles, but to which no serum is added, the second with 0.2 c.c. serum and dose of corpuscles. Shake tubes and incubate for 2 hours. Make a preliminary reading, and read again after tubes have settled for 24 hours in the refrigerator.

(3) The first control should be completely hemolyzed, indicating that a sufficient lytic dose of toxin was employed.

Several strains of *B. tetani* grown in blood agar plates should be tested for hemotoxin production. The most potent strain is used for the inoculation of the broth.

1. What constituent of tetanus toxin has a marked affinity for erythrocytes?
2. Is this agent thermostabile? How can you determine this?
3. What rôle does it play in tetanus intoxication?
4. How is this hemotoxic agent neutralized by tetanus antitoxin?
5. Would antitetanopasmin neutralize the hemotoxic activity of tetanus toxin?
6. Would diphtheria antitoxin neutralize this hemotoxic agent? If not, why not?
7. Explain the mechanism of neutralization of a toxin by antitoxin *in vitro*. Is it the same as that occurring *in vivo?*

References:

Rosenau, M. J., and Anderson, J. F. The standardization of tetanus antitoxin. *U. S. Hyg. Lab. Bull.*, No. 43, 1908, p. 5.

For the production of a potent tetanus toxin, consult:

Dernby, K. G., and Allander, B. Production de la toxine tetanique. *Compt. Rend. Soc. Biol.*, 1921, 85, p. 1181.

Wilcox, H. L. The effect of peptone upon the production of tetanus toxin. *Jour. Bacteriol.*, 1921, 6, p. 407.

EXPERIMENT 96. TETANUS INTOXICATION IN ANIMALS

Use technic in Zinsser *et al.* (ed. 2; New York, Macmillan Co., 1921, p. 154), as follows:

(1) Inject a guinea pig of approximately 250 grams into the thigh of one hind leg with 5 lethal doses of the tetanus toxin.

(2) A similar guinea pig at the same time is injected with twice the dose, i.e., 10 lethal doses of tetanus unit (which has been mixed with one antitoxin unit), the mixture having been allowed to stand at room temperature for 20 minutes.

(3) Grind up aseptically 5 lethal doses of the tetanus toxin with half of one cortex of fresh guinea pig brain, adding salt solution to the amount of 3 or 4 c.c. during the process. This mixture is allowed to stand at room temperature for 2 or 3 hours. It is then centrifugalized and the supernatant fluid injected into a third pig. Do likewise with kidney tissue.

(4) Feed 50 M. L. D. toxin orally.

The pigs are kept under close observation for 6 days.

1. How do you explain the effect of the ground brain and kidney tissue?
2. What is the mode of action and the route of the intoxication?

References:

Wasserman and Takaki. *Berlin. Klin. Wochnschr.*, 1898, No. 1.

Besredka, A. Fixation de la toxine tétanique par le cerveau. *Ann.* de l'Institut Pasteur, 1903, 17, p. 138.

Teale, F. H., and Embleton, D. The paths of spread of bacterial exotoxins with special reference to tetanus toxin. *Jour. Path. and Bacteriol.*, 1919, 23, p. 50.

Tulloch, W. J. Report of bacteriological investigation of tetanus carried out on behalf of the War Office Committee for the study of tetanus. *Jour. Hyg.*, 1919, 18, p. 103.

Buxton and Glenny. Active immunization of horses against tetanus. *Lancet*, 1921, 2, p. 1109.

EXPERIMENT 97. PROTECTIVE POWER OF CL. BOTULINUM ANTITOXIN

Prepare and filter *Cl. botulinus* toxin. Find approximate M. L. D. and, using this unitage, show the effect of early and late administration of antitoxin.

1. Upon what fundamental principles does the success of the antitoxin therapy depend?
2. Why is this antitoxin therapeutically less valuable than diphtheria antitoxin?

References:

DICKSON, E. C., AND HOWITT, B. M. Botulism. *Jour. Am. Med. Assoc.*, 1920, 74, p. 718.

BENGTSON, I. Standardization of B. botulinus antitoxin. *Jour. Am. Pub. Health*, 1921, 11, p. 353.

DICKSON, E. C., AND SHEVKY, R. Studies on the manner in which the toxin of Cl. botulinum acts upon the body. *Jour. Exper. Med.*, 1923, 38, p. 327.

BRONFENBRENNER, J. J., AND SCHLESINGER, M. J. Effect of digestive juices on the potency of botulinus toxin. *Jour. Exper. Med.*, 1924, 39, p. 509.

WEINBERG, M., AND GOY, P. Botulinus anatoxin. *Compt. Rend. Soc. Biol.*, 1924, 91, p. 148.

EXPERIMENT 98. DEMONSTRATION OF CL. BOTULINUM IN FOOD, AND THE EXAMINATION OF SOIL FOR CL. BOTULINUM

Samples of food will be issued for testing. Four samples of soil collected by the student should be examined.

1. Do you often find both A and B types of the organism in the same sample?
2. If you were a health officer, how would you proceed to establish the etiology of a suspected case of botulism?
3. What are the processing regulations for commercially canned foods?

References:

DUBOVSKY, B. J., AND MEYER, K. F. An experimental study of the methods available for the enrichment, demonstration and isolation of B. botulinus in specimens of soil and its products, in suspected food, in clinical and necropsy material. *Jour. Infect. Dis.*, 1922, 31, p. 501.

SCHOENHOLZ, P., ESTY, J. R., AND MEYER, K. F. Toxin production and signs of spoilage in commercially canned vegetables and fruits, inoculated with detoxified spores of B. botulinus. *Jour. Infect. Dis.*, 1923, 33, p. 289.

ESTY, J. R. Heat resistance of B. botulinus spores. *Am. Jour. Public Health*, 1923, 13, p. 108.

DICKSON, E. C., AND SHEVKY, R. Studies on the manner in which the toxin of Clostridium botulinum acts upon the body. *Jour. Exper. Med.*, 1923, 38, p. 327.

BRONFENBRENNER, J. J., AND SCHLESINGER, M. J. The effect of digestive juices on the potency of botulinus toxin. *Jour. Exper. Med.*, 1924, 39, p. 509.

WHEELER, M. W., AND HUMPHREYS, E. M. Isolation of B. botulinus, Type B, from feces by use of blood agar plates in anaerobic jar. *Jour. Infect. Dis.*, 1924, 35, p. 305.

SCHOENHOLZ, P., AND MEYER, K. F. Effect of direct sunlight, diffuse daylight and heat on potency of botulinus toxin in culture mediums and vegetable products. *Jour. Infect Dis.*, 1924, 35, p. 361.

Fifteenth Week

EXPERIMENT 99. WASSERMANN REACTION

Use standard method of Kolmer (ed. 3; Baltimore, W. B. Saunders Co., 1923), third method, p. 481. Read also Coca, A. F., *Essentials of Immunology,* Baltimore, Williams and Wilkins, 1925, pp. 176–186.

1. What is meant by the Neisser-Wechsberg phenomena of complement deviation?
2. What are the advantages of using a polyvalent antigen?
3. Explain the necessity for each control employed in the test.
4. Is the reaction specific?

EXPERIMENT 100. SERUM DIAGNOSIS OF SYPHILIS BY PRECIPITATION, KAHN TEST

Prepare the reagents and set up the test according to Kahn. Test at least 10 negative and 10 positive sera which will be supplied by the instructor.

1. What are the advantages of this test over the Wassermann technic?
2. Does this reaction correlate well with the findings of the Wassermann reaction?

References:

Young, C. C. Kahn precipitation test for syphilis. *Am. Jour. Pub. Health,* 1924, 14, p. 508.

Kahn, R. L. *Serum Diagnosis of Syphilis by Precipitation.* Baltimore, Williams and Wilkins Co., 1925.

EXPERIMENT 101. ORGANOTROPISM

Technic of Kolmer (ed. 3; Baltimore, W. B. Saunders Co., 1923, p. 1156), as follows:

(1) Prepare a 2 per cent alkalinized solution of arsphenamin by dissolving 0.2 gm. in 8.4 c.c. of hot water and adding 1.6 c.c. of N/1 solution sodium hydroxid.

(2) Inject intravenously 4 white rats weighing 100 to 150 grams with the following amounts per 100 grams of weight: 0.4, 0.5, 0.6, and 1 c.c., which administers 80, 100, 120, and 200 mg. of the drug per kilo of body weight.

(3) Prepare a 1:1000 dilution of mercury bichlorid by dissolving 0.1 gm. in 100 c.c. of saline or distilled water.

(4) Inject 4 white rats intravenously with 0.2, 0.4, 0.6, and 1 c.c., which administers 2, 4, 6, and 10 mg. per kilo of body weight.

(5) Observe the animals for 10 days.

1. What is the maximum tolerated dose of arsphenamin? Of mercuric bichlorid?
2. What is meant by toxicity or organotropism?
3. What is meant by *dosis lethalis* and *dosis tolerata?*
4. Which is more toxic, arsenous or arsenic compounds?
5. Are the results of organotropic animal experiments always conclusive evidence regarding the therapeutic value to man?

References:

Moore, Oldershaw and Williams. Toxic effect of heavy metals after subcutaneous inoculation. *Brit. Med. Jour.*, 1913, 2, p. 219.

Felton, L. D., and Dougherty, K. M. Organotropic, bacteriotropic and leucocytropic actions of certain organic chemicals. *Jour. Exper. Med.*, 1922, 36, p. 163.

Dale, H. H. Chemotherapy. *Physiol. Rev.*, 1923, 3, pp. 359–393.

EXPERIMENT 102. CHEMOTHERAPY AND PARASITOTROPISM

Kolmer (ed. 3; Baltimore, W. B. Saunders Co., 1923, p. 1156), as follows:

(1) Infect 4 white rats by injecting each intraperitoneally with 0.2 c.c. of the citrated blood of rat or guinea pig infected with *T. equiderdum* and showing large numbers of the parasites in the tail blood when examined microscopically.

(2) Twenty-four hours later inject No. 1 intravenously with 0.2 c.c. of a 0.5 per cent alkalinized solution of arsphenamin (freshly prepared by dissolving 0.1 g.m. of the powder in 19 c.c. of hot water and adding 1 c.c. of N/1 solution sodium hydroxid) per 100 grams of weight, which corresponds to 10 mg. per kilo of body weight. This amount corresponds to about $\frac{1}{10}$ the toxic dose.

(3) Inject the second rat with 0.2 c.c. of a 1 per cent solution of neoarsphenamin (freshly prepared by dissolving 0.1 gm. in 10 c.c. of sterile water) per 100 gram of weight, which corresponds to 20 mg. per kilo of body weight.

(4) Inject the third rat with 1 c.c. of a 1:5000 dilution of bichlorid of mercuric per 100 grams of weight, which corresponds to 2 mg. per kilo of body weight. This amount corresponds to about ⅓ the toxic dose. The fourth rat is kept as a control.

(5) Examine the tail blood of the 4 rats a few hours later and then at daily intervals.

1. What is meant by parasitotropism?
2. Is arsphenamin trypanocidal? Is mercury bichlorid?
3. What is the purpose of chemotherapy?
4. Discuss the question of chemoreceptors.
5. Discuss 'drug fastness.'

References:

Kolmer, J. A., Schamberg, J. F., Raiziss, G. D. Chemotherapy of trypanosomiasis. *Jour. Infect. Dis.*, 1917, 20, p. 35.

Fourneau, E., Tréfouel, J., and Vallée, J. Chemotherapeutic researches on Bayer "205." *Ann.* de l'Institut Pasteur, 1924, 38, p. 81; also, Trypanocidal action Bayer "205." *Chem. Abstr.*, 1924, 18, p. 3681.

Sixteenth Week

EXPERIMENT 103. PRODUCTION OF PASTEUR RABIES VIRUS

Inject a rabbit subdurally with *virus fixé*. Examine the hippocampus for Negri bodies. Prepare a phenolized fixed virus. Immunize several rabbits and test the immunity by infecting the animals with sheep virus. Discuss the methods used on man and animals.

1. Why is a mutated fixed virus used for immunization?
2. What are the fundamental axioms underlying the Pasteur rabies vaccination?

References:

Stimson, A. M. Facts and problems of rabies. *U. S. Hyg. Lab. Bull.*, No. 65, 1910.

D'Aunoy, Rigney. Antirabic vaccination by means of desiccated virus. *Jour. Infect. Dis.*, 1921, 29, p. 261.

Jackson, Leila. Negri bodies in the salivary glands and other organs in rabies. *Jour. Infect. Dis.*, 1921, 29, p. 291.

Stimson, A. M. Rabies vaccine. *Jour. Am. Med. Assoc.*, 1921, 76, p. 241.

Eichhorn and Lyon. Prophylactic vaccination of dogs against rabies. *Jour. Am. Vet. Med. Assoc.*, 1922, 61, p. 38.

Kelser. A study of rabies from the standpoint of etiology. *Jour. Am. Vet. Med. Assoc.*, 1923–24, 64, pp. 678–689.

EXPERIMENT 104. EXPERIMENTAL HERPES

A strain of Levaditi herpetic virus will be supplied. Etherize a rabbit and infect by corneal scarification. Grind up a portion of the virus brain in 2 c.c. saline and inject subdurally. Observe the symptoms. After death examine brain and spinal cord histologically; search sections and smears for chlamydozoa.

1. Has human encephalitis lethargica ever been experimentally reproduced?
2. What are the cell inclusion bodies seen in brain tissue of 'normal' rabbits.
3. What relationships exist between the diseases of influenza, poliomyletis, and encephalitis lethargica?

References:

LIPSCHÜTZ. Unna's "Balloon degeneration" of prickle cells in light of recent investigation. *Arch. f. Dermatol. and Syph.*, 1921, 4, p. 828.

FLEXNER, S. Epidemic (lethargic) encephalitis and allied conditions. *Jour. Am. Med. Assoc.* ,1923, 81, pp. 1688, 1785.

GOODPASTURE, E. W., AND TEAGUE, O. Experimental production of herpetic lesions in organs and tissues of the rabbit. *Jour. Med. Res.*, 1924, 44, p. 121.

LEVADITTI, C., NICHOLAU, S., AND SCHOEN, R. L'etiologie de l'encéphalite épizootique du lapin dans ses rapports avec l'étude expérimentale de l'encéphalite léthargique. *Ann. de l'Institut Pasteur*, 1924, 38, p. 651.

DOERR, R., AND ZDANSKY, E. A critical experimental investigation of the cause of Herpes febrilis and Encephalitis lethargica. *Ztschr. f. Hyg. und Infectskr.*, 1924, 102, pp. 1–54; also *Abstr. Bacteriol.*, 1924, 8, p. 377.

(Review and Bibliography.) *Med. Sci.*, 1924, 10, p. 355.

EXPERIMENT 105. SMALLPOX. DIAGNOSIS, VACCINATION AND IMMUNITY TEST

All members of the class will be given the intradermal skin test for immunity. Immunize a large white rabbit with smallpox virus and use this animal to diagnose material from suspected cases of the disease. Use the method described by Force, J. N., and Beckwith, H. L., A Laboratory Method for Diagnosis of Smallpox (*Jour. Am. Med. Assoc.*, 1915, 65, p. 588).

1. What is the duration of the immunity of the animals used in the diagnostic test?

2. How would the injection of virulent chicken-pox virus affect the skin test?

3. What are the advantages of the intradermal technic?

4. In judging an immune reaction, why is a control necessary?

5. How is the smallpox virus for human vaccination prepared?

References:

KOLMER. Complement fixation in vaccina and variola. *Jour. Immunol.*, 1916, 1, p. 59.

LEAKE, J. P., AND FORCE, J. N. The essentials of smallpox vaccination. *Public Health Reports* (reprint No. 686), 1921, 36, pp. 1975, 1979.

PHELPS. *U. S. Med. Bull.*, 1924, 20, p. 410.

EXPERIMENT 106. THE WEIL-FELIX REACTION IN THE DIAGNOSIS OF TYPHUS FEVER

Obtain from the instructor a culture of *B. proteus* X_{19} and a strongly agglutinating serum from a case of typhus fever as controls, 10 sera of normal individuals (remainder of Wassermann tests) should be used. As antigen use a 24-hour broth culture diluted to the usual density. Incubate serum dilution at 40° to 45° C.

1. What is the probable relation between typhus fever and this strain of *B. proteus?*

2. Is the Weil-Felix reaction reliable and highly specific?

References:

BENGTSON, I. A. The Weil-Felix reaction as a laboratory test in the diagnosis of typhus fever. *Public Health Reports* (Reprint No. 568), 1919, 34, p. 2446.

MAXCY, K. F., AND HAVENS, L. C. A series of cases giving a positive Weil-Felix reaction. *Am. Jour. Trop. Med.*, 1923, 3, p. 495.

EXPERIMENT 107. BACTERIOPHAGE PHENOMENA

Prepare a bacteriophage active against *B. typhosus.* Obtain a stool specimen of a convalescent case of typhoid fever. If not successful, use the method of Kuttner. Prepare a bacteriophage for a strain of *B. coli* isolated from a case of cystitis. Use the method of Zdansky, Gewinnung von übertragbaren Lysenen für therapeutische Zwecke (*Wien. klin. Wochnschr.*), 1924, 37, p. 501.

1. Does the tissue bacteriophage contain gelatinase?

2. What clinical significance has the presence of a strong "phage" in the stool of a case of typhoid fever?

3. What species of animals yield the strongest bacteriophage principle?

4. Review briefly the theories of the biological nature of the bacteriophage.

References:

HANKIN, M. E. L'action bactéricide des eaux de la Jumna et du Gange—sur le microbe du choléra. *Ann.* Institut Pasteur, 1896, 10, p. 511.

BECKERICH, A., ET HAUDUROY, P. Le bacteriophage de d'Herelle; ses applications therapeutiques. *Jour. Bacteriol.*, 1923, 8, p. 163.

D'Herelle, F. *The Bacteriophage.* Baltimore, Williams and Wilkins, 1922.

Kuttner, A. G. Bacteriophage phenomena. *Jour. Bacteriol.*, 1923, 8, p. 49.

Weiss, E., and Arnold, L. A study of antigenic properties of bacteriophage. *Jour. Infect. Dis.*, 1924, 34, p. 317.

Doerr, R. Bakteriophagen. *Jour. Am. Med. Assoc.*, 1924, 82, pp. 249–250.

Zdansky, E. Gewinniung von übertragbaren Bakteriophagen für therapeutische Zwecke. *Zentralbl. f. d. ges. Hyg.*, 1924, Bd. 9, Heft 2, p. 121.

Hadley, Philip. A method of staining lytic areas produced by the bacteriophage. *Jour. Bacteriol.*, 1924, 9, p. 405.

Kister, T. P. Gegenwärtigen Stand der Bakteriophagen Frage. *Zentralbl. f. d. ges. Hyg.*, 1924, 8.

PART IV

CLINICAL BACTERIOLOGY

PREPARED BY

J. STICKEL

PART IV

CLINICAL BACTERIOLOGY

PREPARED BY
J. STICKEL

I. GENERAL INSTRUCTIONS

Cultures, smears, and other specimens for bacteriological study are usually brought to the laboratory, but in some cases the specimens are collected directly from the patient by the bacteriologist. Each specimen should be labeled with the patient's name and location and should be accompanied by the regulation laboratory request blank, giving the information indicated on this slip. When the laboratory examination has been completed, the findings are recorded in the space provided on the blank, and the complete report is sent either to the patient's ward, or, in the case of a private patient, to the physician in charge. A duplicate of this report is kept on file in the laboratory.

On working days the regular time for receiving specimens in the laboratory is between the hours of 9:00 A.M. and 4:30 P.M. Outside of these regular hours and on holidays, special arrangements must be made for the examination of specimens. A section in the laboratory ice box is equipped with the various culture media recommended in this section of the syllabus. This store of media is available at all times of the day and night.

The use of aseptic methods in collecting specimens is necessary. The instruments and receptacles used in collecting the material must be sterile. Care must be taken to prevent the contamination of the specimen by skin and body organisms, either from the patient or the individual who is collecting it. After collection, the material must not be allowed to stand exposed to contamination by air organisms or be permitted to touch any unsterile object, but must be placed immediately in a sterile, closed receptacle.

All specimens should be examined and cultured as soon after collection as possible. Immediately after the inoculation of the media, cultures should be placed in the incubator (unless they are to be kept at a temperature other than body temperature). In cases where the laboratory work cannot be done at once, materials which are not liable to contain saphrophytic bacteria should be kept in the incubator until such time as they can be cultured. Specimens like sputum and feces which contain saprophytes are usually kept in the ice box until the time they are examined.

LIST OF DISEASES REPORTABLE BY LAW
STATE OF CALIFORNIA, 1925

Anthrax
Beri-beri
Botulism
Cerebrospinal Meningitis (Epidemic)
Chickenpox
Cholera, Asiatic
Dengue
Diphtheria
Dysentery
Encephalitis (Epidemic)
Erysipelas
Flukes
Food Poisoning
German Measles
Glanders
Gonococcus Infection*
Hookworm
Influenza
Jaudice, Infectious
Leprosy
Malaria
Measles
Mumps
Ophthalmia Neonatorum
Paratyphoid Fever
Pellagra
Plague
Pneumonia
Poliomyelitis
Rabies
Rocky Mountain Spotted (or Tick) Fever
Scarlet Fever
Smallpox
Syphilis*
Tetanus
Trachoma
Tuberculosis*
Typhoid Fever
Typhus Fever
Whooping Cough
Yellow Fever

Quarantinable Diseases

Cerebrospinal Meningitis (Epidemic)
Cholera, Asiatic
Diphtheria
Encephalitis (Epidemic)
Leprosy
Plague
Poliomyelitis
Scarlet Fever
Smallpox
Typhoid Fever
Typhus Fever
Yellow Fever

* Use special card enclosed in envelope.

State Hygienic Laboratory Card for:

REPORT OF A CASE OF COMMUNICABLE DISEASE

Mail This Card Immediately. No Postage Required.

Use This Side to Report ONE CASE ONLY.

Place and date ....., 192

Disease ..... Onset .....

Name of patient ..... Age .....

Address of patient .....

School attended ..... Employed at .....

Probable source of infection .....

Vaccination history:

If smallpox, when successfully vaccinated .....

If diphtheria, how many doses toxin-antitoxin .....

When .....

If typhoid, how many doses ..... When ..... Where .....

Remarks .....

I first saw this patient at ..... o'clock on .....

Signature of physician .....

Address of physician .....

To be mailed to:

ASSISTANT COLLABORATING EPIDEMIOLOGIST,

United States Public Health Service,

....., California

UNIVERSITY OF CALIFORNIA

MEDICAL SCHOOL UNIVERSITY HOSPITAL HAHNEMANN HOSPITAL

LABORATORY CONSULTATION

To Dr. ..... Hospital / Clinic No. .....

Patient ..... Room / Ward No. .....

Essential Clinical Data

Material from

Consultation requested

Charge to { teaching / patient

....., 192..... Dr. .....

Laboratory No. ...., 192.....

Laboratory Findings:—

Dr. .....

II. STUDY OF SPECIMENS FROM THE EYE, EAR, MOUTH, NASAL SINUSES, RESPIRATORY TRACT AND PLEURA

Eye

Collection of material.—Collect the specimen by means of a sterile swab. Obtain the material only from the area affected. If the surface is ulcerated or membranous, it is well to remove the superficial material with a sterile swab and by means of another sterile applicator take the smear from the deepest surface.

Ear

Collection of material.—Cleanse the external ear with warm water and soap. Remove the excess of secretion with a sterile swab. Collect the exudate from the infected tissue.

Mouth

Collection of material.—The mouth is rinsed out with sterile water. Obtain the material by rubbing a sterile cotton swab gently but firmly over the affected area only.

Nasal Sinuses

Collection of material.—The specimen is collected by the surgeon.

External Nares

Collection of material.—Cleanse the nasal orifice with soap and water. Pass a sterile cotton swab through a nasal speculum. Rub lightly the lower turbinates, septum, and posterior pharyngeal wall for the collection of nasal secretion. If there is a special lesion to be examined, obtain material only from the area affected. If the surface is ulcerated or membranous, it is well to remove the superficial material and by means of a fresh sterile swab take the smear from the deepest surface.

Posterior Naso-Pharynx

Collection of material.—Use a West tube and proceed as described on page 167.

Throat

Collection of material.—No attempt should be made to collect the material shortly after the application of strong disinfectants to the throat. Cultures are often more successful if the throat is first

cleansed with a spray of sterile, physiological salt solution. In the case of infants, any food or vomitus present should be removed by means of a sterile swab.

The patient is placed in a good light, and, if a child, properly held. The tongue is depressed by means of a sterile tongue depressor, while a sterile cotton swab is passed into the pharynx (without touching the tongue or other parts of the mouth) and rubbed gently but firmly against any lesion visible on the tonsils or in the pharynx. It is well to take another swab from the tonsils and pharynx in general.

Sputum

Collection of material.—The specimen is best collected in the morning. The sputum which the patient first raises in the morning should be discarded. The desirable material is that which comes from deep in the bronchi, uncontaminated by saliva, and discharges from the throat and naso-pharynx.

(*a*) Adults. The patient's teeth and mouth are cleaned by means of a sterile toothbrush and sterile water. After this cleansing the patient is instructed to swallow two or three mouthfuls of sterile water. He is then told to "cough from his boots" and expectorate into a sterile wide-mouth bottle or Petri dish.

(*b*) Infants. The child's mouth must be held wide open by means of a sterile tongue blade. Coughing is induced by tickling the pharynx with a sterile cotton swab. The exudate which is coughed up is caught on this same swab, care being taken to prevent the contamination of the swab by mouth organisms.

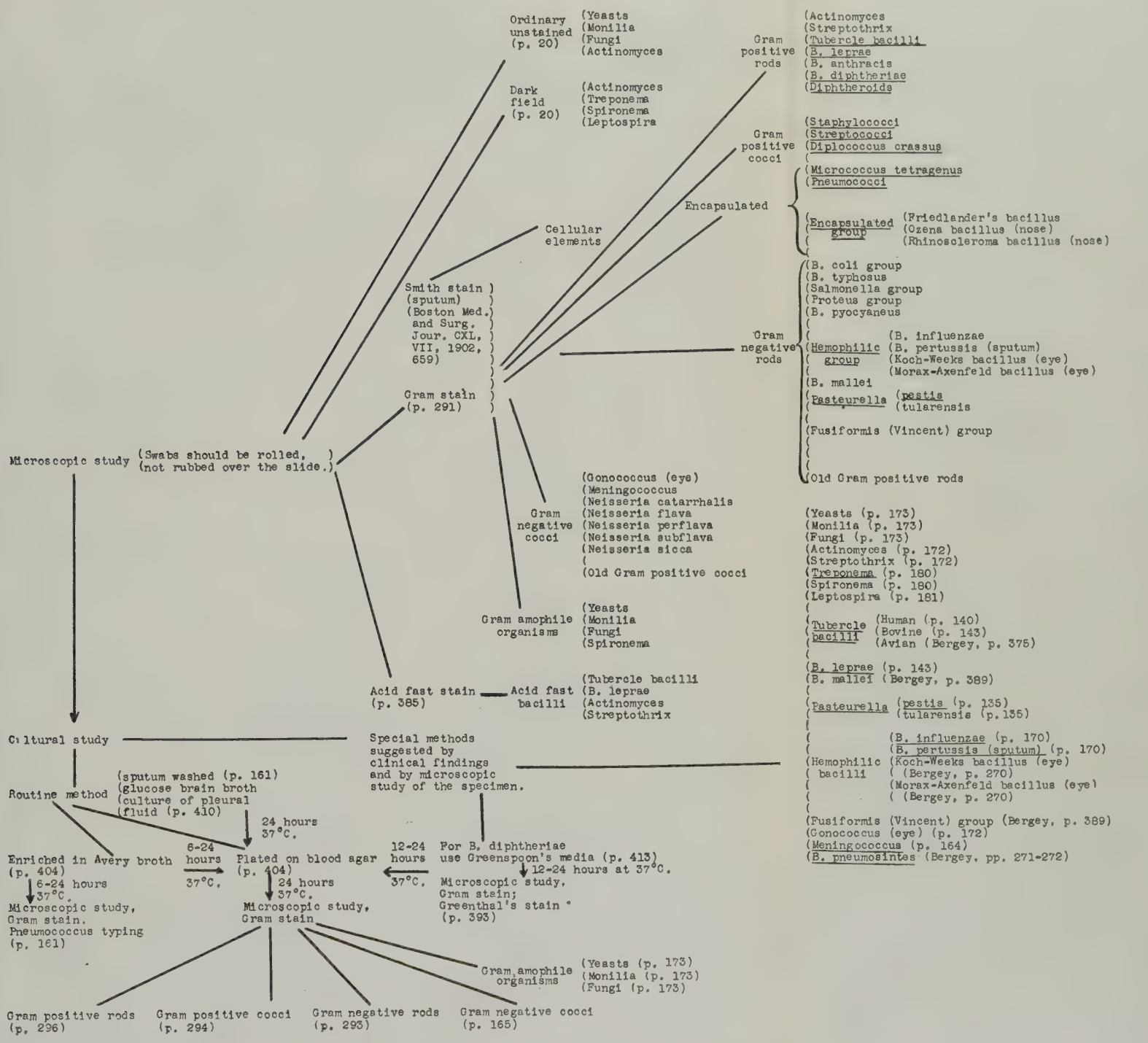
The specimen should be examined immediately. If this is not possible, it should be kept at ice-chest temperature until it is examined. (Naturally, this would not be done if one suspected the sputum of containing organisms which might be destroyed by chilling.)

Lung Juice

Collection of material.—This is an emergency procedure and is done only when it is impossible to obtain a specimen of sputum. Aspirate 0.5 c.c. of sterile physiological salt solution by means of a sterile 10 c.c. record syringe to which has been fitted a sterile 19 gauge needle. Disinfect the patient's skin by painting with tincture of iodine. Thrust the needle about two inches into the lung in the suspected pneumonia area. Inject the saline without introducing air. Two or three drops, occasionally more, of blood-stained fluid can be withdrawn.

Wash the lung fluid from the syringe into a tube of sterile Avery broth and incubate at 37° C.

TABLE 13.—Examination of Specimen from the Eye, Ear, Mouth, Nasal Sinuses, Respiratory Tract, and Pleura.



III. SPECIMENS FROM THE GENITO-URINARY TRACT

Urine from the Bladder

(*a*) Male. Wash the glans and meatus with soap and water and a 2 per cent lysol solution.

The three glass test.—Instruct the patient to pass his urine into three sterile glasses, interrupting the flow of urine in transferring from one glass to another. The three specimens thus obtained represent:

(1) Bladder urine (less sediment) plus washing of urethra.

(2) Bladder urine (less sediment) uncontaminated. Use for cultures.

(3) Bladder urine plus sediment and prostatic secretions.

(*b*) Female. The external parts are cleansed with soap and water and a 2 per cent lysol solution. The urethral orifice is further cleansed with 2 per cent lysol followed by sterile water, about ten pledgets being saturated with each.

Use sterile catheters and lubricate with sterile oil (liquid petrolatum). The catheter is handled either with sterile gloves or sterile forceps. Collect urine in two or three sterile test tubes. The last one should contain 20 c.c. for bacteriological study.

All specimens of urine must be examined immediately. Determine the P_H and study a drop of the uncentrifugalized urine in an unstained preparation (cells, motility of bacteria). When alkaline, examine for *Micrococcus ureae,* etc.

Urine from the Ureters

These specimens are collected by the surgeon in the operating room.

Urethral and Vaginal Discharges

Collection of material.—Cleanse the external parts with soap and water and a 2 per cent lysol solution.

(*a*) Adult males. In cases of acute infection, the secretion can be collected directly from the urethral orifice by means of a sterile platinum wire loop. In chronic cases, have the patient present himself with full bladder. Cleanse the glands and meatus with soap and water and a 2 per cent lysol solution. Allow the patient to void mate-

rial from the anterior urethra into one sterile test tube, and then, while pressing the prostrate and posterior urethra, into a second tube. Secretions may be stimulated by massage and by injections of silver nitrate. The sediment obtained by centrifulgalization at high speed for five minutes is used for laboratory study.

(*b*) Adult females. Secretions from the cervix and uteri are collected by inserting a sterile uterine tube, the labia being held well apart by an attendant wearing sterile rubber gloves. Such a tube can be made from ordinary glass tubing having a diameter of about 5 mm. The end for insertion is bent at an angle of about 5 degrees at a distance of about 12 cm. from the tip. The tip is smoothed in the flame.

Material is also collected from the urethral orifice by means of a sterile swab or platinum wire loop.

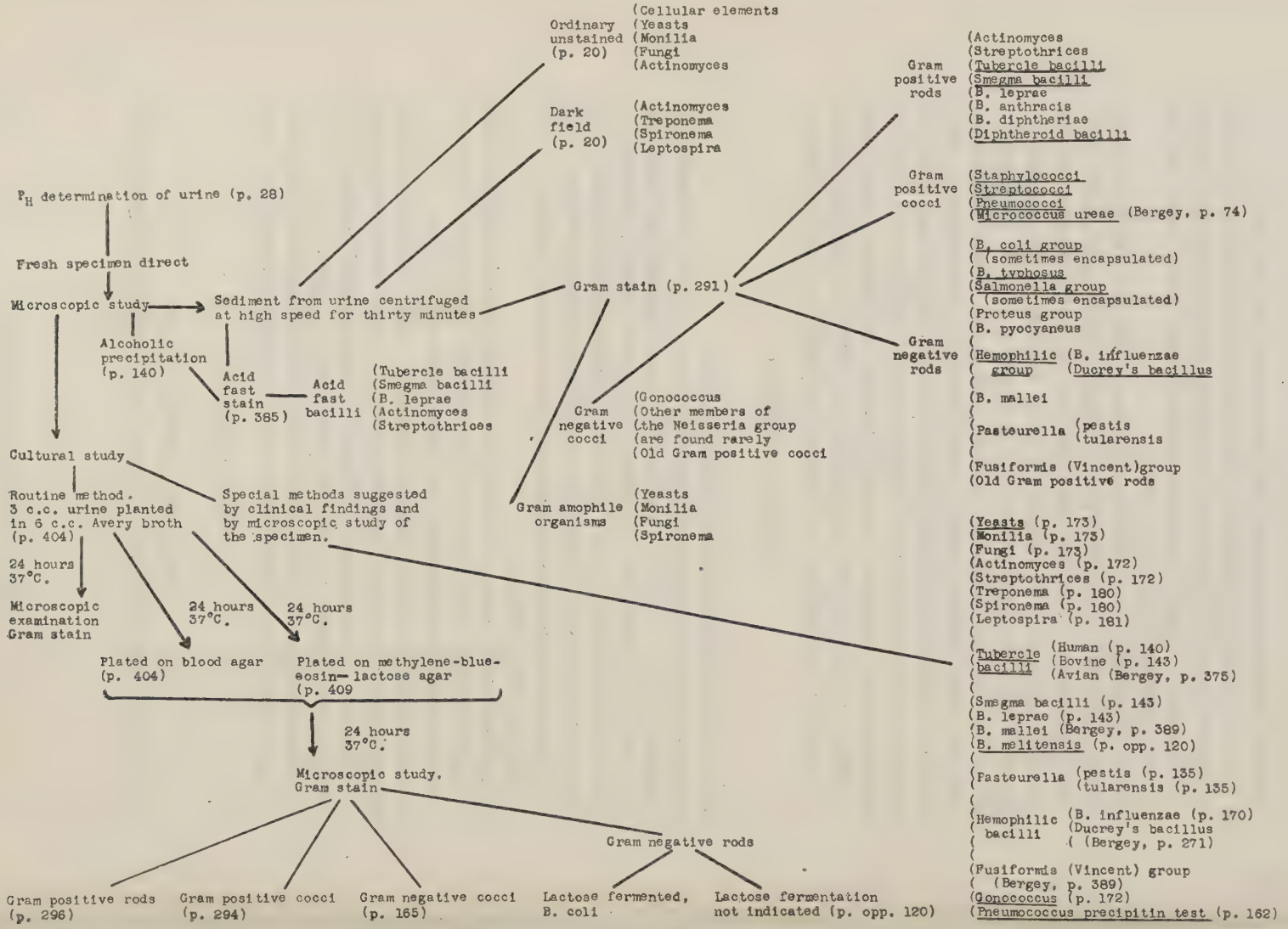
(*c*) Female children. For obtaining the vaginal material, the labia are held well apart by an attendant wearing sterile rubber gloves. A sterile slender cotton swab is used which passes easily into the vagina without touching any external part. The swab is rubbed gently about the vaginal wall.

In cases of infantile vaginitis, vaginal washings often yield better results than the swabs. By means of a sterile syringe, fill the vagina with a 1:4000 solution of mercury bichloride in sterile normal salt solution. Recover the fluid in a sterile dish and transfer to a sterile centrifugal tube. Centrifuge at high speed for 15 minutes and examine the sediment.

Prostatic Secretion

Collection of material.—The external genitalia are washed with soap and water and a 2 per cent lysol solution. The urethra is flushed out with sterile physiological salt solution. The prostate is massaged and the fluid is collected through a sterile urethroscopic tube lubricated with sterile liquid petrolatum.

TABLE 14.—Examination of Specimen from the Genito-Urinary Tract.



IV. STUDY OF SPECIMENS FROM THE GASTRO-INTESTINAL TRACT AND PERITONEUM

Gastric Contents

The material is collected by means of a sterile stomach tube. For the technic of using such a tube, see Webster, *Diagnostic Methods* (ed. 7, Philadelphia, P. Blakiston's Sons and Co., 1923), p. 56.

Ordinarily there are few organisms in the stomach, although it is possible to find a variety of organisms which are present as a result of swallowing mouth and throat secretions. These organisms can be demonstrated microscopically and in cultures (opp. p. 276).

When lactic acid is present in the stomach (stasis and carcinoma of the stomach), organisms which thrive well in an acid medium are present in large numbers.

These organisms include certain of the sarcinae (Bergey, p. 71) and the yeasts (p. 88), and the so-called Boas-Oppler bacillus. The Boas-Oppler bacillus is a long, slender, non-spore-bearing, gram-positive rod. It is a member of the group of aciduric bacilli or lactobacilli (Bergey, p. 243), and can be cultured by the same methods used for culturing the lactobacillus acidophilus (p. 210). These organisms are rare in non-malignant disease of the stomach.

Duodenal Contents

Collection of material is by means of an Einhorn tube. A regular breakfast is allowed and after about two hours the metallic sinker, which has been chilled in ice water, is put in the patient's mouth and a small drink of water given, with the patient sitting up in bed; as soon as the patient swallows the sinker he (or she) lies down on the right side while waiting for the tube to descend to the duodenum(third mark). On an average a wait of about four or five hours is necessary. At the end of three hours, the tube is 'primed' by aspiration with a syringe, which usually produces a small amount of whitish mucus, acid to litmus. This procedure often establishes a siphon, and in another hour yellowish-green fluid, neutral or alkaline to litmus, may be seen dropping out of the tube. Further suction gives from 10–20 c.c. of genuine duodenal contents.

Laboratory study.—Examine microscopically and plate out on blood agar plates, and methylene blue-eosin-lactose agar plates. Plant 4 c.c. in a tube of Avery broth; incubate 24 hours and examine by means of Gram-stained smears and plate out as directed above.

Feces

Collection of material.—

(*a*) Adults. The stool is collected in a sterile bed pan. By means of a sterile tongue blade, a portion of the specimen is placed in a sterile Petri dish or wide-mouthed bottle and taken immediately to the laboratory.

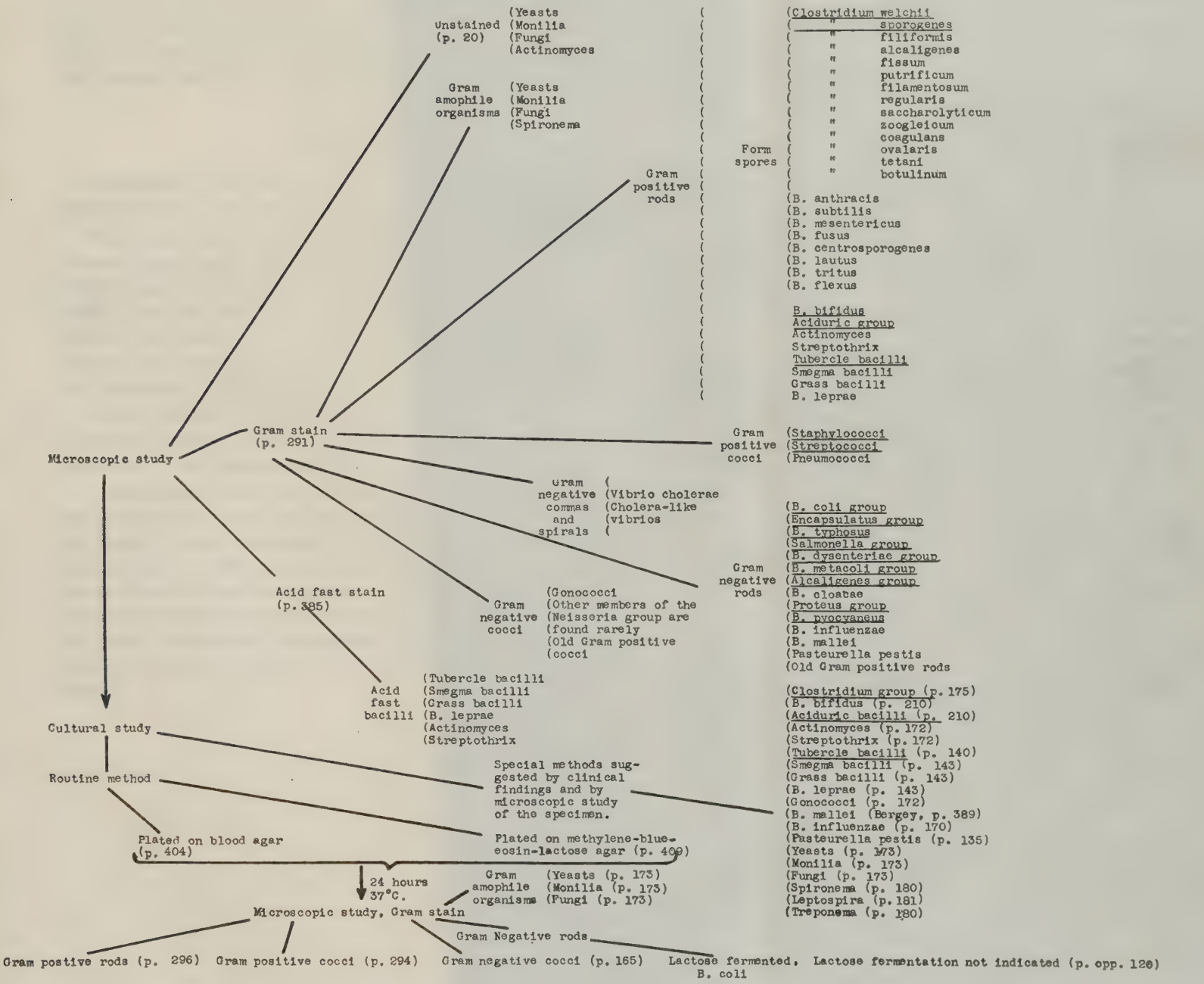
(*b*) Infants. Rectal tubes are prepared in the following manner: Take glass tubing having a diameter of 4 m.m. and cut it into 6-inch lengths. Smooth the ends in the flame. Plug one end of each tube with cotton. Place each piece of tubing (plugged end up) inside of an ordinary test tube, 150 mm. in length. Plug the test tube with cotton and sterilize in the dry air sterilizer.

After cleansing the anus, the end of the glass tube which has not been plugged with cotton is lubricated with sterile vaseline, and inserted into the rectum to a distance of an inch or an inch and a half. It is desirable to collect material sufficient to rise to a height of 2 inches in the tube.

Stool cultures.—Feces may contain any microöganisms with which the individual may come in contact. The bacteria and higher vegetable forms mentioned in table 15 include those which occur with constancy in certain specific infectious diseases, or else inhabit the intestinal tract of the normal individual. Certain of these bacteria, which are the cause of specific intestinal infections, must be emphasized together with the methods for their cultivation.

Disease Produced		Causative Organism	Bacteriological Methods
Typhoid fever		B. typhosus	P. 117.
Paratyphoid fever		Salmonella group	P. 117.
Food poisoning	Gastro-intestinal form	Salmonella group B. proteus B. coli group	P. 117.
	Nervous form	Clostridium botulinum	Demonstration of botulismus toxin in suspected food stuff (p. 179). Cultivation of clostridium botulinum (p. 178).
Bacillary dysentery		B. dysentery group Salmonella group Morgan's bacillus	P. 126.
Cholera		Vibrio cholerae	P. 127.

TABLE 15.—Examination of Specimen from the Intestinal Tract and the P eritoneum.



In certain metabolic disturbances, it is worth while to determine the type of chemical action which the intestinal bacteria are producing. Cultures are made in carbohydrate and in protein media. The biochemical reactions produced in these substances indicate whether the changes produced by the combined action of the organisms which make up the intestinal flora are predominantly fermentative or proteolytic. The technic for this test is given on page 210.

Peritoneal Fluid

Collection of material.—For the technic of performing a paracentesis, see Webster, *Diagnostic Methods,* ed. 7, p. 791.

Have ready at the patient's bedside a bottle containing 100 c.c. of glucose brain broth, warmed to a temperature of 37° C. Introduce into this medium 10 c.c. of the freshly collected peritoneal fluid. Mix rapidly and thoroughly by rotating the bottle of broth between the palms of the hands. Incubate at 37° C. for 24 hours. Examine microscopically by means of a Gram-stained smear and plate out on blood agar plates and methylene blue-eosin-lactose agar plates.

Collect 10 c.c. of the fluid in a test tube containing 10 c.c. of ammonium oxalate solution. Centrifuge at high speed for one hour. Examine the fluid microscopically and make cultures.

The remainder of the fluid is collected in sterile flasks. If coagulation takes place, grind up a portion of the clot with a sterile mortar and pestle; make microscopic preparations and cultures. When an examination for tubercle bacilli is to be made, antiforminize (p. 141) the clot from the entire specimen. If there is no coagulation, make use of the sediment from a portion centrifuged at high speed for one hour. The larger the quantity centrifuged the better; 400 c.c. can easily be used by balancing two pairs of 100 c.c. centrifuge tubes against each other.

V. STUDY OF SPECIMENS FROM THE SKIN AND SUBCUTIS AND OF TISSUES, EXUDATES AND FLUIDS REMOVED DURING SURGICAL OPERATION

Skin and Subcutis

Collection of material.—

Avoid rough maniplation of the tissues. Cleanse the area surrounding the lesion with soap and water, followed by the application of 70 per cent alcohol on a sterile cotton swab. In the case of vesicles, pustules, and abscesses, the top is lifted off with the top of a sterile scalpel. Collect the exudate either on at least three sterile swabs, or by aspirating with a capillary pipette.

The exudates from wounds, ulcers, and necrotic and gangrenous lesions are collected by using several sterile swabs.

Curettings from scaly skin lesions are used for examinations.

Specimens Removed During Surgical Operation

For the method of culturing tissues see page 73.

In collecting exudates and fluids, collect as large a quantity of the specimen as possible.

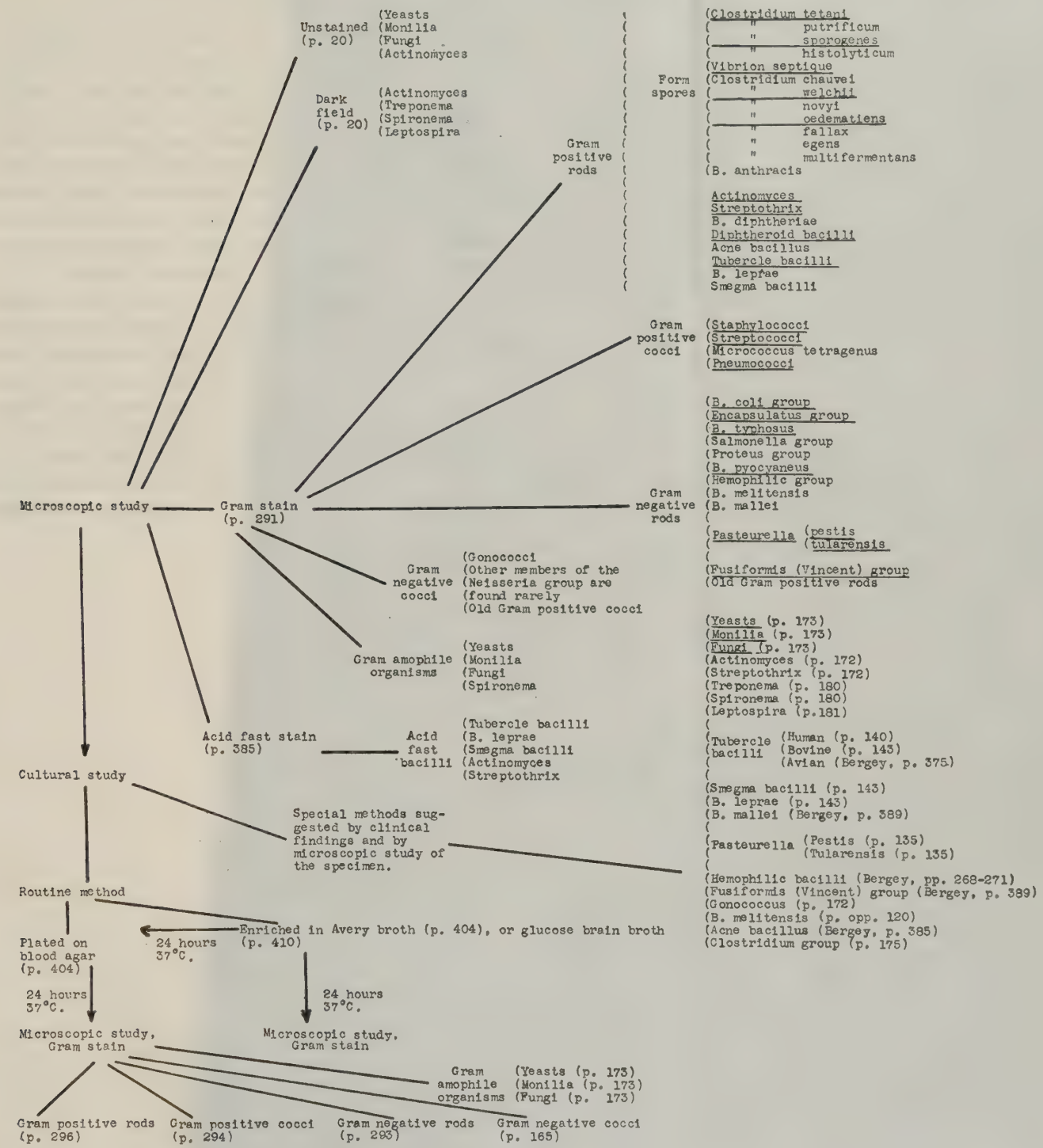
Tooth Cultures

Collection of material.—

(*a*) Acute abscesses.—Clean the mouth carefully by means of a sterile alkaline boric acid solution and a sterile toothbrush. Rub the gums with 70 per cent alcohol, and paint with crystal violet-brilliant green solution (1 gram each in 100 c.c. of 50 per cent alcohol). Through a small incision introduce deeply into the abscess a fairly coarse Pasteur pipette or with a sterile needle and sterile Luer syringe aspirate the pus. If it is not possible to make an examination immediately, seal both ends of the pipette and preserve in a cool dark place.

(*b*) *Chronic abscesses of teeth which are to be extracted.*—Remove all the tartar or other deposits from the teeth, clean the mouth with an antiseptic spray, and scrub carefully the mucous membrane of the offending tooth with 70 per cent alcohol. Isolate the tooth by carefully packing both sides with gauze; apply a small amount of acetone

TABLE 16.—Examination of Specimen from the Skin and Subcutis and of Tissues, Exudates, and Fluids Removed During Surgical Operation.



to dry the tooth and membrane; saturate the tooth and surrounding tissues with iodine or crystalviolet-brilliant green solution. After the ligamentum circulare is cut, either apply iodine or cauterize the edge of the mucous membrane. Extract the tooth with a freshly boiled forcep. The tooth is then superficially flamed by holding it with a sterile forcep over an alcohol burner, avoiding exposure of the apex. With sterile Rongeur forceps, clip off the apex and drop into deep tubes containing liquid media (No. 4 or No. 25). With sterile instruments curette the alveolar socket from which the tooth was extracted, and place the granulation tissue in glucose brain broth. For details consult Appleton, Clinical dental bacteriology, *Dental Cosmos,* 1924.

(*c*) *Chronic abscesses and granulation tissues collected during apicoectomy.*—Pack off the operative field of the gums. Apply in the following succession: hypochlorite solution, 70 per cent alcohol, acetone, crystalviolet-brilliant green solution. It is always well to control this method of disinfection by rubbing a sterile cotton swab over the treated area and placing the swab immediately in broth. When the operation is performed with the necessary precautions of asepsis, the amputated root and the curetted granulation tissue when removed are not contaminated by oral microörganisms and may be dropped into fluid culture media.

VI. STUDY OF SPECIMENS FROM THE CIRCULATORY SYSTEM AND THE PERICARDIUM

Blood Serum for Immunological Tests

Collection of blood.—See page 122.

The following serological tests are important in diagnostic laboratory work:

Wasserman test for syphilis (p. 310).

Test for agglutinins for bacteria of the coli-typhoid-paratyphoid group (Widal test, p. 123).

Test for agglutinins for bacilli of the dysentery group (p. 126).

Test for streptococcus agglutinins (p. 235).

Test for agglutinins for *B. melitensis* (p. 134).

Complement fixation test for tuberculosis (p. 249).

Blood Culture

For taking a blood culture it is convenient to have a tray upon which the following articles are assembled:

Tincture of iodine.
Sterile applicators.
Sterile gauze.
Rubber tubing for tourniquet.
Clamp for tourniquet.
Adhesive tape.
Two bottles, each containing 100 c.c. of glucose brain broth (No. 24).
One bottle, containing glucose veal broth with a Ph of 7.6 (No. 29).
Sterile 20 c.c. Luer syringe and a sharp, sterile hypodermic needle, 19-gauge and 1.5 inches long.
Alcohol lamp.
Matches.
Enamelware pint measure containing 3 per cent cresol solution.

Collection of material.—

(*a*) *Adults.*—A contaminated blood culture is of no value. Therefore, careful technic must be used in obtaining the blood. Expose the patient's arm and cleanse the area over the veins at the elbow with green soap and warm water, followed by 70 per cent alcohol and ether. When the skin is perfectly dry, disinfect by painting with tincture of iodine twice at intervals of one minute. It is undesirable to remove

the iodine with alcohol, but this should be practiced if the patient is hypersensitive to iodine. Instead of disinfecting with tincture of iodine, the following method may be used. After washing the skin with soap and hot water, apply a solution prepared by dissolving 0.5 gram of brilliant green and 0.5 gram of crystal violet in 100 c.c. of 50 per cent alcohol. Cover the painted area on the skin with dry, sterile gauze. Take the blood 15 minutes after applying this solution.

Under ordinary circumstances, sterile blood cultures can be taken without wearing rubber gloves and without the preparation of a sterile field about the site of puncture, if the operator's technic is good. Do not contaminate the disinfected area before the blood is withdrawn. If it is necessary to palpate the vein, do so with the iodinized finger. Apply a rubber tourniquet to the middle of the upper portion of the arm, taking care not to shut off entirely the pulse at the wrist. Instruct the patient to extend the arm fully, to open and close the hand several times, and then to make a fist. Usually the veins will become prominent and can be easily entered.

The blood is obtained by means of a clean, dry, sterile, all-glass syringe (a 20 c.c. Luer is recommended) to which is attached a large sharp, dry, sterile needle. Avoid contaminating the plunger of the syringe, the inside of the syringe, and avoid touching the needle for at least an inch from the tip. Insert the needle into the most prominent vein, taking care that the needle lies flat and enters the vein almost parallel to the skin, thus avoiding passing through the vein.

After taking the blood, loosen the tourniquet, withdraw the needle, and cover the puncture at once with several layers of sterile gauze which is pressed down to stop the flow of blood. This gauze is removed after about 5 minutes. Clean the patient's arm with 70 per cent alcohol on sterile cotton; touch the needle puncture with a drop of tincture of iodine on a small, sterile cotton swab; cover the area with several layers of sterile gauze and fix them in position by means of adhesive strips. This dressing may be removed on the following morning.

If blood cannot be obtained by direct puncture of the vein through the skin, a small incision may be made (under ethyl chloride anesthesia) obliquely to the course of the vein, which is then exposed to blunt dissection. This procedure is carried out aseptically like any minor surgical operation. After the blood is withdrawn from the exposed vein, the incision is closed and surgically dressed, and care is taken to prevent an infection of the patient.

(*b*) *Infants.*—The skin is prepared as outlined above. In young children it is usually impossible to obtain blood from the elbow veins and a puncture of the longitudinal sinus or of the jugular vein is

often resorted to. If this is undesirable, the lobe of the ear, tip of the finger, or toe, may be pricked and the drops of blood collected in the culture media.

Inoculation of culture media.—

(*a*) *Routine method.*—Three bottles of broth are inoculated. The bottles have a capacity of 150 c.c. and each contains 100 c.c. of medium; two of them contain glucose brain broth and the third contains glucose veal broth having a P_H of 7.6. Previous to sterilization, these bottles are provided with paper caps so as to insure the sterility of the necks of the bottles. Warm these bottles to 37° C. Withdraw 15 c.c. of blood from the patient and plant 5 c.c. in each of the three bottles. Clean the needle and syringe before the blood clots. In order to prevent the spread of infectious material, this is best done by alternately aspirating and forcing out of the syringe a 3 per cent cresol solution until the blood is all removed from the syringe. The syringe and needle should be left submerged in the cresol solution for at least 30 minutes before proceeding with their cleaning.

Mix the blood and broth together as soon as possible by rotating the bottle between the palms of the hands. This will prevent the coagulation of the blood. A blood culture in which the blood has clotted is of almost no value. Place the bottles in the incubator before the cultures have had time to cool to room temperature. It is well to place them in a dish of water having a temperature of 37° C. immediately after inoculation and thus transport them to the incubator.

The cultures are examined after incubation over-night. Microscopic examinations are made by means of Gram-stained smears and subcultures are made on the surfaces of blood agar (No. 6), and of methylene blue-eosin-lactose agar plates (No. 21). If no growth has been discovered after keeping the bottles in the incubator for seven days they are discarded and considered as being sterile.

PERICARDIAL FLUID

Collection of material.—

For the technic of performing a pericardicentesis see Webster, *Diagnostic Methods*, ed. 7, p. 791.

Have ready at the patient's bedside a bottle containing 100 c.c. of glucose brain broth (No. 24), warmed to a temperature of 37° C. Introduce into this medium the freshly collected pericardial fluid. Mix rapidly and thoroughly by rotating the bottle of broth between the palms of the hands. Incubate at 37° C. and proceed as in the examination of blood cultures.

Organisms Which Are Isolated from Blood and Pericardial Fluid

(*a*) *By the routine method.*—

Bacilli of the colon-typhoid-paratyphoid and encapsulatus group (opp. p. 120).

Proteus group (opp. p. 120).

B. pyocyaneus (p. 90).

Bacilli of the hemophilic group (p. 169).

B. melitensis (opp. p. 120).

B. mallei (Bergey, p. 389).

Pasteurella pestis (p. 135).

Pasteurella tularensis (p. 135).

B. anthracis (pp. 109, 299).

Micrococcus tetragenus (p. 149).

Staphylococci (p. 149).

Streptococci (p. 150).

Pneumococci. A pneumococcus typing can be done directly with this broth culture (pp. 155, 308).

Meningococci (p. 164). Never permit the cultures to become cold.

Yeasts (p. 174).

Monilia (p. 174).

Fungi (p. 174).

Actinomyces (p. 172).

Streptothrix (p. 172).

(*b*) *Special methods.*—

Plate cultures are made for estimating the number of bacteria which can be cultivated from each cubic centimeter of the patient's blood. Two or three tubes of glucose veal infusion agar are melted by boiling in an enamel-ware cup. Just before taking to the patient's bedside, the water in this boiler is cooled to 50° C.; when the cultures are made its temperature should be 42° C. Add 1 c.c. of blood, or more if indicated, to each tube of agar. Mix the blood with the agar by rotating betwen the palms of the hands. Flame the lip of the tube and pour the agar into a sterile Petri dish. Leave the plates undisturbed on the table until the agar has completely hardened. Then invert the Petri dishes and incubate at 37° C. The colonies are counted after 48 hours.

Bile medium for B. typhosus.—Add 10 c.c. of blood to a bottle containing 25 c.c. of sterile ox-bile. Incubate at 37° C. Examine as outlined under routine method.

Trypsinized broth.—The addition of trypsin to the culture media is said to destroy the bactericidal properties of the blood. Ten cubic centimetres of trypsin should be added to each 90 c.c. of broth.

"Liquor Pancreatica," prepared by the Digestive Ferments Co., may be used. This is put up in sterile ampules. Aseptic precautions must be used in adding the trypsin to the broth, and the broth must not be heated afterwards, as heat destroys the action of the trypsin. Trypsin broth keeps its strength for at least 4 weeks.

Anaerobic cultures.—Broth cultures made as outlined under the routine method are stratified aseptically with vaseline and incubated in anaerobic jars. Subsequent subcultures are made as described on page 175.

Oxalated and citrated blood.—Blood not added to culture media may be prevented from clotting, either by mixing it with an equal portion of the ammonium oxalate solution described on page 380, or by adding it to a tube containing sterile sodium citrate solution. (For each 10 c.c. of blood use 0.2 c.c. of a solution containing 10 per cent sodium citrate and 0.85 per cent sodium chloride.)

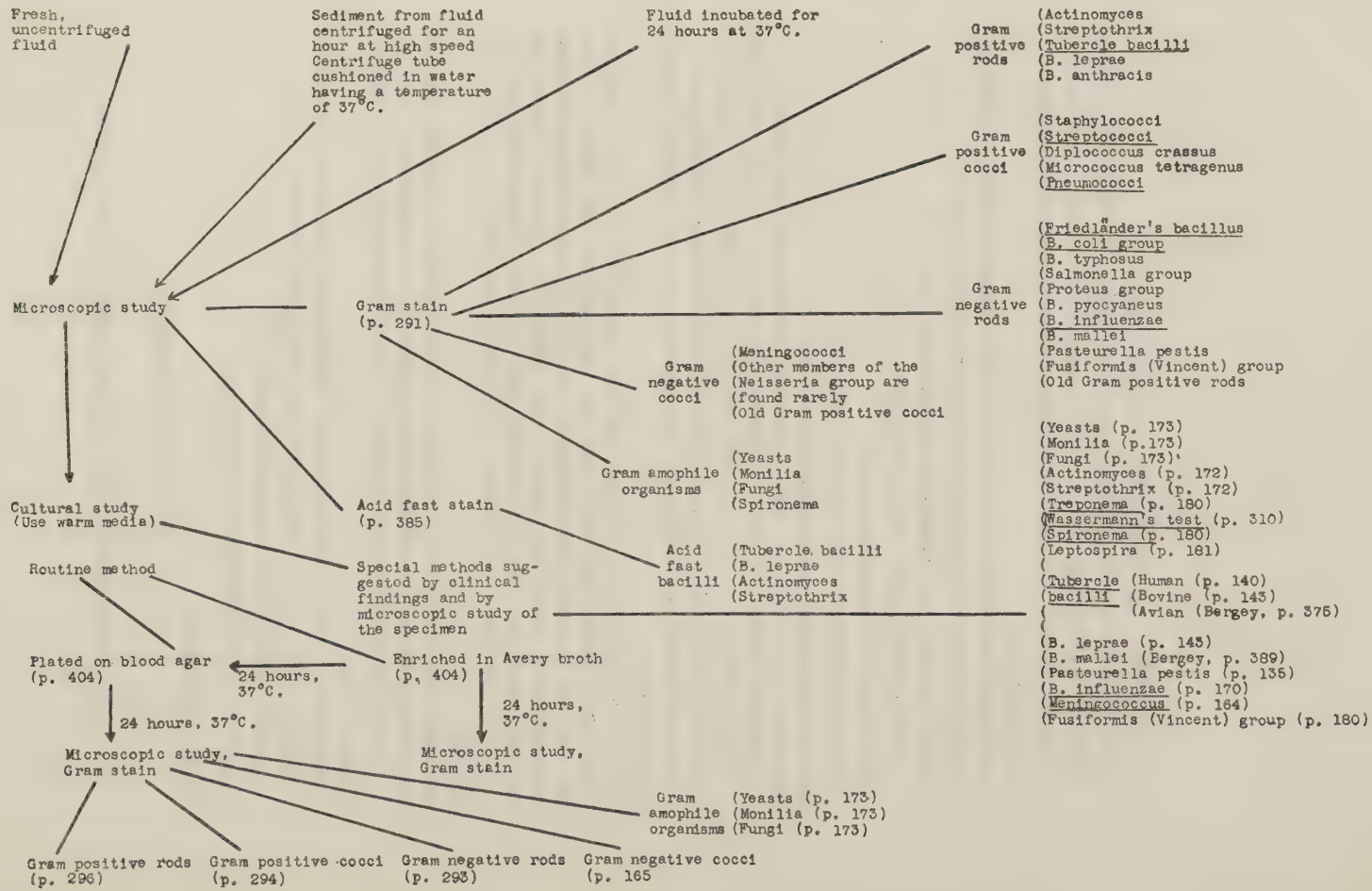
Blood cultures for the isolation of gonococci.—Proceed as in the routine method, but subculture the broth on slants of testicular agar (No. 72). For the identification of gonococci, see page 165.

VII. STUDY OF MATERIAL FROM THE CENTRAL NERVOUS SYSTEM

Collection of material.—

Fluid from the cerebral ventricles, and pus from brain abscesses, are collected by the surgeon in the operating room. For the technic of making a lumbar puncture, see Webster, R. W., *Diagnostic Methods,* p. 811. Before collecting the specimen, there should be ready an enamel cup containing 3 sterile test tubes immersed to about one-half their height in water having a temperature of 37° C. The fluid should be collected in these tubes and immediately afterward the tubes replaced in the warm water. Cerebrospinal fluid for bacteriological examination should never be permitted to become cold, and should be examined as soon after it is collected as possible. The specimens should not be shaken more than can be avoided, since this is liable to interfere with the formation of a fibrin clot or web.

TABLE 17.—Examination of Cerebrospinal Fluid.



VIII. PREPARATION OF BACTERIAL VACCINES

A. Plain Vaccines

Materials Required.—

Flasks of sterile physiological salt solution.

Sterile plugged 10 c.c. volumetric pipettes.

Cylinder of 3 per cent cresol solution for disinfecting pipettes.

Sterile, plain 15 c.c. centrifuge tubes plugged with corks covered by a layer of heavy muslin.

Vaccine filters. These are prepared by placing a thin layer of absorbent cotton and gauze in a small glass funnel (about 5 cm. in diameter). They are either wrapped in paper or are placed in a metal container and sterilized by dry heat.

Sterile filters may also be prepared by heating a test tube (150 × 18 mm.) to red heat about 7 cm. from the plugged end. The tube is withdrawn from the flame and slowly drawn out to make a stem about 8 cm. in length. Seal off the stem and after the tube has cooled place the filter pad in the constricted portion of the tube. Replace the plug, wrap the filter tube in heavy paper or place several in a plugged metal or glass container. Sterilize at 15 pounds pressure for 30 minutes.

Sterile, plugged Pasteur pipettes, 20 cm. long. 3 to 4 c.c. capactiy. Always use a rubber suction bulb on the end of such a pipette when handling fluids.

Vaccine centrifuge tubes. For standardizing small amounts of vaccine use the Hopkins model, for large quantities ordinary 15 c.c. centrifuge tubes graduated to 0.1 c.c. may be used. These are plugged with corks covered by a layer of heavy muslin and sterilized by dry heat.

Sterile, plugged 100 c.c. volumetric pipettes.

Sterile 100 c.c. centrifuge tubes.

Sterile, plugged 10 c.c. pipettes graduated to 0.1 c.c.

Sterile plugged 1 c.c. pipettes graduated to 0.01 c.c.

Sterile bottles, 30 c.c. capacity, containing 10 small beads.

Water bath with water to a depth of 15 cm. in which a constant temperature can be maintained.

Solution composed of 8 per cent tricresol in physiological salt solution.

Solution composed of 0.25 per cent tricresol in physiological salt solution.

A pure culture of each organism which is to be used in making the vaccine.

Culture media. The following are recommended:

Organisms	Media for growing the vaccine	Media for Sterility Tests	
		Solid	Liquid
Staphylococci	Plain agar slants	Plain agar slant	Plain broth
Streptococci	Dextrose veal broth in flasks or bottles.	Blood agar slant	Avery broth
Pneumococci	Dextrose veal broth in flasks or bottles.	Blood agar slant	Avery broth
Gonococci	Testicular agar slants	Testicular agar slants	Testicular broth
M. catarrhalis and similar Gram negative diplococci.	Glycerin veal agar slants	Glycerin, veal agar slants	Avery broth
B. proteus. B. pyocyaneus. Bacilli in coli-typhoid-paratyphoid-dysentery group.	Plain agar slants	Plain agar slants	Glucose broth
B. influenzae	Chocolate (cooked blood agar slants).	Chocolate agar slant	Avery broth

NOTE: Sterility tests for anaerobic organisms are always made in glucose veal broth stratified with petrolatum.

Procedure.—This is carried out in a room which is clean and free from dust and air currents. Inoculate the culture media and incubate for from 24 to 48 hours. Make Gram stains. Examine under the microscope to make sure that the growth is composed of the desired organisms and is free from contamination. If the bacteria are growing on solid media, by means of a sterile 10 c.c. volumetric pipette add sterile physiological salt solution to the tubes, thus washing off the organisms and making a heavy suspension of them in the saline. (Immediately after use all pipettes are placed in a cylinder of cresol solution to become disinfected.) Place a sterile 15 c.c. centrifuge tube in a test-tube stand, remove the cork, place one of the vaccine filters in the mouth of the tube. With a sterile 10 c.c. volumetric pipette take up the bacterial suspension and strain it through the filter into the centrifuge tube until about 10 c.c. are carried over. Replace the cork in the centrifuge tube; centrifuge at high speed for 30 minutes; pour off the supernatant fluid; add 10 c.c. of fresh saline and repeat the centrifugalization and pouring off of the supernatant fluid. Resuspend the bacteria in 10 c.c. of saline and by means of a sterile Pasteur pipette transfer to a vaccine centrifuge tube.

If the bacteria are growing in liquid culture media, by means of a sterile 100 c.c. pipette transfer the culture to a sterile 100 c.c. centrifuge tube. Centrifuge at high speed for 30 minutes; pour off the

supernatant fluid; add saline to the tube; centrifuge; pour off the salt solution; add fresh saline; centrifuge; pour off the saline; resuspend the organisms in salt solution and filter into a vaccine centrifuge tube.

After transferring the organisms to the vaccine centrifuge tube, throw down and pack the bacteria by centrifuging at a speed of 2800 revolutions per minutes for 30 minutes. Then by means of a sterile pipette remove the supernatant fluid and bacterial sediment to the 0.05 c.c. mark. With a sterile graduated pipette add sufficient sterile saline, to bring the volume to 5 c.c. thus making a 1 per cent suspension. If more or less than 0.05 c.c. of packed bacteria are used, add the amount of salt solution necessary to give a 1 per cent suspension.

By means of a sterile Pasteur pipette, remove the bacterial suspension from the centrifuge tube and place it in a small sterile bottle containing a few small beads. Shake until a perfectly homogenous suspension results, taking care not to allow the fluid to touch the cotton plug in the bottle.

By means of a sterile pipette, transfer the vaccine to a sterile test tube, which is then sealed in a blast flame. Submerge the test tube containing the bacterial suspension, in a water bath having a temperature of 53°–60° C. (depending on the organism). Heat for 1 hour. Remove the tube from the water bath. By means of sterile pipette add 1 drop of the vaccine to each of the culture media recommended for the sterility test. Incubate for 48 hours. If no growth occurs the vaccine is accepted for use; but if growth takes place in these control tubes, it must be heated again and fresh cultures made to test its sterility.

After inoculating the control tubes, add tricresol to the vaccine. Shake vigorously the bottle containing the 8 per cent tricresol mixture. By means of a sterile graduated 1 c.c. pipette, add 0.25 c.c. of 8 per cent tricresol for each 10 c.c. of vaccine. Label the vaccine container with the patient's name, the name of the organism, the concentration of the suspension, and the date. Keep in the ice box until used.

The standardization of the vaccine is based upon the bacterial content of 1 per cent suspensions as given in the following table (Hopkin's method):

Staphylococci	10	billions per c.c.
Streptococci	8	billions per c.c.
Gonococcus	8	billions per c.c.
M. catarrhalis	8	billions per c.c.
Pneumococcus	2.5	billions per c.c.
B. typhosus	8	billions per c.c.
B. coli	4	billions per c.c.
B. influenzae	20	billions per c.c.

Dilutions are made from the 1 per cent stock suspension. To prepare a given dilution, determine the volume of stock solution which contains the desired number of organisms to make the quantity ordered. Measure this amount of stock vaccine by means of a sterile graduated pipette; place it in a sterile bottle, and add sufficient quantity of the 0.25 per cent tricresol solution to bring about the required dilution.

Vaccines for intravenous injection are diluted in the same way, except that sterile physiological salt solution is used as a diluting fluid instead of the 0.25 per cent tricresol solution.

The vaccine bottle is capped with a sterile rubber cap, thin enough to be punctured by a hypodermic needle. Label, giving the patient's name, type of the bacteria, concentration, percentage of tricresol, and the date. Keep in the ice chest until used.

As regards the dosage, no fixed rules can be given. In acute infections the dose should be small and may be repeated frequently; in chronic infections, larger doses should be given at longer intervals. Children tolerate relatively large doses, but the dosage should depend on the weight and not the age of the child.

The following is a list of the ordinary subcutaneous doses for adults for various vaccines:

Staphylococcus aureus	100,000,000	to	1,000,000,000
Staphylococcus albus	200,000,000	to	1,000,000,000
Streptococcus pyogenes	25,000,000	to	200,000,000
Gonococcus	25,000,000	to	200,000,000
Typhoid bacillus	250,000,000	to	1,000,000,000
Colon bacillus	100,000,000	to	1,000,000,000

B. Sensitized Vaccines

Proceed as in making a plain vaccine. Before heating the 1 per cent suspension mix it with an equal volume of a sterile inactivated highly potent immune serum, specific for the organisms used in making the vaccine. The mixture is gently agitated at room temperature for about 5 hours. Centrifuge the emulsion and wash three times with sterile saline. After the final washing, make up the bacterial suspension with sterile saline, shake to insure the breaking up of agglutinated clumps. Heat in the water bath and proceed as stated above.

Slightly larger doses of sensitized vaccine are used than corresponding doses of plain vaccine.

TABLE 18

Gram Negative Rods which Grow on Plain Blood Agar Plates

Type of colony	Morphology	Media for subculturing colony	Tentative classification	Key for complete identification
Large, opaque, smooth edges	Short, plump, (sometimes encapsulated).	Plain agar slant (No. 57)	Coli-typhoid-paratyphoid-Proteus group.	Page opp. p. 120.
Large, opaque, viscid, smooth edges	Short, plump, encapsulated	Plain agar slant	Encapsulatus group	Page opp. p. 120.
Large, opaque, spreading, dirty, discoloration of media, disagreeable odor.	Short, plump	Plain agar slant	No pigment. Proteus	Page opp. p. 120.
			Green pigment. B. pyocyaneus	Bergey (p. 123).
Very small, moist	Very tiny	Chocolate agar slant (No. 7)	Hemophilic group	Page 169. Bergey, pp. 268–271
Small, yellow, round, opaque	Small, stains irregularly	Glycerin-veal agar (No. 32)	B. mallei	Bergey (p. 389).
Small, translucent, irregular borders	Ovoid, bi-polar, pleomorphic	Plain agar slant	Pasteurella group. B. pestis	Page 134.
Small, round, regular borders	Small, oval	Plain agar slant or blood agar	Br. melitensis	Page opp. p. 120.

TABLE 19

Gram Positive Cocci which Grow on Plain Blood Agar Plates

Type of colony	Morphology	Media for subculturing colony	Abbreviated identification methods			Tentative classification	Key for complete identification
Large, opaque, circular, smooth edges.	Grouped irregularly; not encapsulated.	Loeffler's serum (No. 48).	Pigment (pp. 47, 91)	Colorless growth		Staphylococcus albus and others.	Page 149.
				Orange colored growth		Staphylococcus aureus.	
				Lemon colored growth		Staphylococcus citreus.	
Small, raised, opaque	Biscuit-shaped pairs, not encapsulated.	Loeffler's serum				Diplococcus crassus.	Stitt, p. 96.
Glistening, circular, smooth edges, viscid.	Grouped in tetrads; encapsulated.	Loeffler's serum				Micrococcus tetragenus.	Page 149.
Moist, often viscid, smooth edges 1 mm. or more in diameter, green zone surrounds colony.	Occur in pairs; sometimes in chains; elongate and often lancet-shaped; capsules usually demonstrable.	Avery broth (No. 4); poured blood agar plate (p. 154).	After 24 hours at 37° C., in the poured blood agar plate, each colony is surrounded by a zone in which the red blood cells have undergone greenish discoloration and partial hemolysis.	Soluble in ox-bile (p. 156).	Agglutinated by Type I pneumococcus antiserum	Pneumococcus, Type I.	Page 156.
					Agglutinated by Type II pneumococcus antiserum	Pneumococcus, Type II.	
					Agglutinated by Type III pneumococcus antiserum	Pneumococcus, Type III.	
Small, green zone surrounds colony.	Occur in pairs and chains; often elongate; capsules not demonstrable.	Poured blood agar plate.			Not agglutinated by either of the antisera for the three fixed types of	Pneumococcus, Group IV.	
				Not soluble in ox-bile.		Streptococcus, alpha type.	Page 150.

TABLE 19—(*Concluded*)

Type of colony	Morphology	Media for subculturing colony	Abbreviated identification methods	Tentative classification	Key for complete identification
Small, surrounded by a hazy, colorless zone.	Occur in pairs and chains; often elongate; capsules not demonstrable.	Avery broth; poured blood agar plate.	After 24 hours at 37° C., in the poured blood agar plate each colony is surrounded by a colorless zone of partially dissolved red blood cells.	Streptococcus, alpha prime type.	Page 150.
Small, surrounded by a clear, colorless zone.	Occur in pairs and chains; often elongate; capsules not demonstrable.	Avery broth; poured blood agar plate.	After 24 hours at 37° C., in the poured blood agar plate each colony is surrounded by a colorless zone of completely hemolyzed red blood cells.	Streptococcus, beta type.	
Vary greatly in size; no change in the media surrounding the colony.	Occur in pairs and chains; often elongate; capsules not demonstrable.	Avery broth.	Formation of definite chains in Avery broth.....	Streptococcus, gamma type.	

TABLE 20

Gram Positive Rods which Grow on Plain Blood Agar Plates

Type of colony	Morphology	Spores	Motility	Media for subculturing colony	Abbreviated identification methods	Tentative classification	Key for complete identification
Large with irregular margins.	Straight, slender; sometimes form chains.	Spores formed (pp. 42, 394).	Not motile (pp. 19, 42, 84).	Plain agar slant (No. 57)	Pathogenic for mice (p. 109) . .	B. anthracis	Bergey, p. 308
					Not parhogenic for mice.....	Saprophytic organism..	Bergey, pp. 309–310
			Motile.....	Plain agar slant..	.....	Saprophytic organism..	Bergey, pp. 278–308
Small	Pleomorphic; often contain metachromic granules (pp. 384, 393). Parallel and digital grouping.	Not formed.....	Not motile	Greenspoon's medium (. 34).	Toxic for normal guinea pigs. Not toxic for guinea pigs immunized against diphtheria toxin (p. 145).	Diphtheria bacillus. .	Page 147.
					Not toxic for normal guinea pigs.	Diphtheroid bacili.....	Page 147.

PART V

STATE HYGIENIC LABORATORY TECHNIC

PREPARED BY

W. H. KELLOGG

PART V

STATE HYGIENIC LABORATORY TECHNIC

PREPARED BY
W. H. KELLOGG

I. Anthrax

A diagnosis of anthrax is given only when microscopical and cultural examination are confirmed by animal inoculation.

If the specimen received is dried blood it is emulsified in salt solution; if an ear, a vein is dissected out so as to obtain the blood; if tissue, other than an ear, the surface is seared, an incision made so as to avoid surface contamination, and a small particle removed and emulsified in salt solution.

Microscopical examination.—Direct smears from blood or tissue stained with methylene blue show bamboo-like rod-shaped bacilli.

Cultural examination.—A tube of nutrient broth is inoculated with the blood or tissue emulsion obtained as stated above. Three tubes of agar are inoculated with the same material, making 3 dilutions (loop method), and plates poured. After 24 hours' incubation the broth is examined for the typical stringy cotton-like growth in the bottom of the tube and the agar plates for the fur-like filamentous colonies.

Animal inoculation.—One to 2 c.c. of a saline emulsion of blood or tissue are inoculated subcutaneously into a guinea pig. If the guinea pig dies, cultures are made from the heart blood into broth and agar as above. After 24 hours' incubation these cultures are examined, smears are made from typical colonies, and if Gram positive, square-ended bacilli in chains with spore form are found, a diagnosis of anthrax is given (p. 109).

II. Botulism

The technic used in this laboratory for the identification of *Cl. botulinus* is as follows:

In examining fruits and vegetables the juice is centrifuged and the sediment is examined for spores. In examining organs of chickens, tie off the crop and gizzard, add the contents to sterile water, and incubate

to develop spores. Glucose broth, brain and glucose agar, are inoculated with large amounts of the suspected material; immediately before inoculating, both suspected material and culture media are boiled for at least 10 minutes. The fluid media, broth and brain, can be quickly cooled by plunging into tap water, but the agar is cooled only to about 45° C. so that the material can be thoroughly mixed with the fluid agar. Petrolatum is used for sealing the media to insure anaerobic conditions. All culture tubes are incubated at from 26–28° C.

The points essential for the identification of *Cl. botulinus* are the following:

(1) Demonstration of large Gram-positive bacilli with rounded ends and subterminal spores.

(2) Anaerobic growth of characteristic appearance in glucose agar cylinders with formation of gas and fragmentation of the medium.

(3) Blackening of brain medium.

(4) Characteristic growth in glucose infusion broth with the production of a virulent toxin demonstrable in filtered broth by subcutaneous inoculation into guinea pigs. One cubic centimeter of the broth is used. The animal dies in 24 hours. There is first a collapse and the legs are sprawled straight out. At autopsy the intestines are deeply injected.

Glucose agar cultures are examined for characteristic colonies in 48 to 72 hours, but if negative, are not discarded for at least 2 weeks. Glucose broth cultures are allowed to incubate for about 1 month before they are discarded. Cultures of *Cl. botulinus* have the characteristic odor of rancid butter, caused by the production of butyric acid.

For immediate identification of the toxin in suspected foods, the following method is used: If no fluid is present, an infusion is made with water or salt solution and filtered. If fluid is present, it is filtered out alone or mixed with the infusion of the solid portion and is injected subcutaneously into each of 3 guinea pigs. One pig is given at the same time a protective dose of Botulinus antitoxin type A, one is given type B, and the third is given both types. According to the type of toxin present, one pig or the other of the two given one type of antitoxin will die. If the material contains both types of toxin in lethal amount, both pigs will die, the one receiving both types of antitoxin surviving.

III. Diphtheria

Swabs from the throat and nose are sent to the laboratory for diphtheria examinations. The swabs are planted on Loeffler's blood serum and after about 16 hours' incubation smears are made, air-dried, fixed by heat, and stained with the following stain:

Toluidin blue	1 gm.
Alcohol, 95 per cent	10 c.c.
Glacial acetic acid	25 c.c.
Distilled water	500 c.c.

The fixed smears are immersed in this stain in staining jars for at least 5 minutes. There is no danger of over-staining. A positive diagnosis is made on finding granular, barred, or solid diphtheria bacilli. The granular types show purple metachromatic granules with a blue body; the barred types show blue bars with deeper staining poles, and the solid types show blue bodies with characteristic morphology. Practically all organisms except diphtheria bacilli and certain cocci take this stain very faintly, so that the diphtheria organisms stand out against a faint blue background.

TESTING THE VIRULENCE OF CULTURES MORPHOLOGICALLY POSITIVE FOR DIPHTHERIA

Many bacteriologists formerly considered it necessary to isolate a pure culture by plating, and to inoculate into a guinea pig, death of the animal with characteristic lesions being required. The use of mixed cultures, i.e., suspension of the growth on the diagnostic slant, for subcutaneous inoculation, was first suggested by N. E. Wayson. If properly controlled by inoculating at the same time another pig that has been previously immunized with 200 or 300 units of diphtheria antitoxin, the method is satisfactory and is approved by the State Hygienic Laboratory. Death of the test animal within 96 hours and survival of the control is essential, and the dead pig must be autopsied and the diagnosis confirmed by the finding of characteristic pathological changes. These consist of large, cherry-red adrenals, necrosis at the site of inoculation, and a surrounding gelatinous edema. If the control animal also dies, some other pathogenic organism is present, and the issue is clouded. A diagnosis of diphtheria may still be made, provided the signs above mentioned are unmistakable in the test pig and absent in the control; otherwise, the result is inconclusive. If the test pig lives, the result, of course, is negative.

A virulence test without the use of an immunized control is absolutely unreliable and will not be approved.

The method in use at the Hygienic Laboratory is that of Force and Beattie. This method is the intradermal inoculation into the skin of a white guinea pig of a 0.2 c.c. of a salt solution suspension of the original or 'field' culture. The density of the suspension is roughly adjusted to about that of triple typhoid vaccine, but may be varied somewhat according to the number of diphtheria bacilli as compared to the associated organisms. White pigs are selected, because the test depends on the local reaction produced by the injection, which is made into and not under the skin, the same as the Schick test. At the same time an injection is made into the skin of another guinea pig which has received, preferably a few hours earlier, a dose of 200 or 300 units of antitoxin.

The principal advantage of this method is that several tests can be placed on one test animal and one control animal, whereas the subcutaneous method requires two pigs for each test.

The technic of the test is as follows: Clip and shave the sides of the guinea pig. Wash down the Loeffler slant with sufficient normal salt solution to give the desired suspension. Make the inoculation with a 1 c.c. Luer or Record syringe and 26-gauge short bevel needle, using a separate syringe and needle for each culture. If more than one test is applied to one pig, space them an inch apart.

When several virulent cultures are placed on one animal, there is some possibility of its dying before full development of the reaction; it is therefore a good plan to give a following dose of antitoxin. If 100 units of antitoxin are given to the test pig 5 hours after the tests are put on, it will not interfere with the development of the skin lesion, but will protect the animal against death from the excess of toxin that might be developed in the skin lesion. A very good working schedule is to give the antitoxin to the control pig at 9 A.M., place the tests at noon and give the following dose of antitoxin to the test pig at 5 P.M. The tests can usually be read in 48 hours, but sometimes it is necessary to hold them longer. A positive reaction is characterized by a central necrotic area surrounded by alternating zones of red and white, giving the so-called target appearance. This target appearance, while characteristic, is not always well marked. It does not require much experience, however, to recognize the necrosis of diphtheria toxin. Following the diffuse redness immediately resulting from the injection, a central white blanching develops in a few hours, which usually persists as long as 48 hours, after which it rapidly changes to a black central scab. The control animal either shows nothing or a simple inflammation without necrosis. Sometimes a superficial abscess

is caused by pyogenic organisms in the culture, but this, while yellowish white, is easily distinguished from the toxin necrosis, which shows no pus and which is also usually more indurated than the other condition. The inoculation should be placed in the same relative location on both test and control animal so as to facilitate comparison.

Guinea pigs cannot be used a second time for the reason that immunity may develop, rendering them unfit for test animals, while the antitoxin sensitizes them against subsequent doses of the same substance.

It is suggested that workers study the reaction in guinea pigs by making a few tests with diphtheria toxin. The State Hygienic Laboratory will be glad to furnish, upon application, some toxin of the proper strength for this purpose.

References:

Wayson, N. E. The laboratory diagnosis of diphtheria. Pub. Health *Reports*, 31, p. 3113 (November 10, 1916).

Force, J. N., and Beattie, M. I. An intracutaneous test for the virulence of diphtheria bacilli in field cultures. *Am. Jour. of Hygiene*, 2, p. 490 (Sept., 1922).

IV. Dysentery

From a specimen of feces to be examined for dysentery bacilli, mucous flakes are fished and dallied out on plates of eosin, methylene blue, lactose agar made according to the following formula:

Meat infusion (10 lbs. of veal to 15 litres of distilled water)	
Peptone	1 per cent
Sodium chloride	0.5 per cent
Agar (sheet)	2 per cent

Adjust first to +0.5 to phenolphthalein, then to 6.6 on the hydrogen ion scale. ph 6.6

Add to the sterilized medium:

Lactose	1 gm. per 100 c.c.
0.2 per cent aqueous solution eosin	2 c.c. per 100 c.c.
0.5 per cent aqueous methylene blue	2 c.c. per 100 c.c.

Sterilize 5 minutes in the Arnold, cool before pouring plates.

Transfer a small flake of mucus to the first plate, add about 2 drops of saline, spread over the entire surface with a dalli; then carry on to 3 or 4 plates, using the same dalli. Incubate 24 hours. Dysentery colonies are transparent, moist, with a slightly flattened surface and have a faint purple color. Coli colonies have deep purple opaque centers.

The suspicious colonies are planted onto agar slants and the following sugars: lactose, mannit, dextrose, and maltose.

Dysentery bacilli give acid in dextrose, but not in the other sugars.

Paradysenteries give the following reactions:

	Hiss-Russell	*Flexner*
Lactose	no acid	no acid
Saccharose	no acid	acid
Mannite	acid	acid
Dextrose	acid	acid
Maltose	no acid	acid

A Gram negative non-motile bacillus is grouped according to the fermentation reactions and agglutinated with the specific serum.

V. Gonococcus Infection

Smears received at the laboratory are stained by Gram's method. If two smears from the same source are received one is stained with methylene blue for morphology.

The formulae of the Gram's stain and technic used are as follows:

Carbol gentian violet:

Saturated alcoholic gentian violet	10 c.c.
Phenol 2 to 2.5 per cent	100 c.c.

Gram's Iodine:

Iodine	1 gm.
Potassium iodid	2 gms.
Distilled water	300 c.c.

Counterstain:

0.5 per cent aqueous solution of safranin.

Stain the smears with carbol gentian violet 1 minute; wash off excess of stain with water, add Gram's iodine for 1 minute, decolorize with 95 per cent alcohol until purple color disappears. Counterstain with safranin 30 seconds.

Positive laboratory findings are based upon the presence of intracellular Gram negative diplococci.

Inconclusive findings are based upon the presence of extracellular Gram negative diplococci or upon the presence of very many pus cells.

VI. Hookworm and Other Intestinal Worms

For routine dignosis of suspected intestinal worms the centrifuge method is most satisfactory. A piece of feces about the size of a walnut is stirred thoroughly in about 5 times its bulk of filtered water. A cyclone mixer of the type used in soda fountains may be employed

to insure thorough mixing. This mixture is then filtered through a a piece of cheese cloth to remove coarse particles. The residue on the cheese cloth should be examined for tapeworm proglottids or whole round worms. The mixture is then centrifuged to get rid of the lighter particles, oils, etc. The centrifuging should be repeated 3 times. After each time the liquid should be poured off and the residue mixed with filtered water. From 10 to 15 seconds at a speed of 1000 revolutions per minute is enough to throw down the eggs.

This residue, which contains parasite eggs mixed with the heavier particles, must be spread out on a slide for study in water sufficient that the material will not be too dense for microscopic examination. A medium power of the microscope is used and a mechanical stage, so that all parts of the preparation can be examined. Two or three preparations, depending on the size of the cover glasses, should be examined from each case before making the diagnosis.

VII. Intestinal Protozoa

The diagnosis of intestinal protozoa is made by examination of feces. If fecal matter can be obtained in fresh condition the simple smear method is best. A small piece of feces is smeared on a slide with a toothpick or similar instrument. Three slides should be made for each case. If the stool is solid at least one slide should be made from slime on its surface. The material on the slide can be thinned with filtered water to the proper consistency for examination. The preparation is then covered with a thin cover glass and examined with the high power of the microscope. The use of the mechanical stage makes it possible to cover all parts of the field. Intestinal flagellates and amoebae or their cysts can be distinguished in such a preparation. Diagnosis by this method is safe only with fresh material, since parasitic protozoa disintegrate rather quickly.

When fecal specimens are to be sent to the laboratory from a distance a special outfit is sent out; namely, a small wide-mouthed bottle partly filled with Schaudinn's fluid (1 part absolute alcohol and 2 parts saturated solution of mercuric chloride) and 6 cover glasses. To make the preparation on these cover glasses a small amount of the fresh stool is placed between two of the cover glasses so that it will spread out in a thin film over their surfaces; the cover glasses are then separated and dropped into the Schaudinn's fluid, which has been heated to 50–60° C. Six cover glasses with material from 3 different parts of the stool should be prepared in this way. The cover glasses are left in the fixing fluid until shipment, but should be shipped immediately. In the laboratory these fixed smears are first washed in 50

per cent alcohol and are then transferred to a 70 per cent alcohol to which a sufficient amount of tincture of iodine has been added to give it the color of port wine. The smears are then run through the grades of alcohol down to water, placed in 1 per cent iron alum for from 1 to 2 hours and stained in 0.5 per cent aqueous solution of iron haematoxylin. If the stain is too heavy the preparations may be decolorized in the 1 per cent iron alum. They are then washed, run up through the grades of alcohol to 100 per cent, and then to xylol, and finally mounted in balsam. This gives a permanent preparation which can be examined with the oil-immersion lens if necessary.

VIII. Leprosy

Smears for microscopical examinations for leprosy are stained in the following manner:

Flood the slide with carbol fuchsin and heat gently over a small flame until it steams for about 3–5 minutes.

Decolorize about 30 seconds with acid alcohol.

Wash in water and counterstain with Loeffler's methylene blue.

The formulae for the stains used are as follows:

Carbol fuchsin:	
Saturated alcoholic basic fuchsin	10 c.c.
Phenol 5 per cent	90 c.c.
Acid alcohol:	
Nitric acid concentrated	3 c.c.
Alcohol, 95 per cent	97 c.c.
Loeffler's methylene blue:	
Saturated alcoholic methylene blue	30 c.c.
1/10,000 solution potassium hydroxide	100 c.c.

IX. Malaria

Blood smears to be examined for malaria are stained with Wright's stain. At least one-half hour, and usually much longer, is spent searching for malarial parasites before a negative report is given.

X. Meningitis (Epidemic)

Spinal fluids sent to the laboratory for examination for epidemic meningitis are centrifuged, the sediment smeared on slides, air dried, fixed with heat, and stained by Gram's method. Gram negative intracellular diplococci are searched for and, if found, a tentative positive report is given. If the spinal fluid is suitable it is plated on sheep serum-dextrose agar and examined by the same procedure as nasopharyngeal smears, the technic of which is as follows:

Nasopharyngeal swabs are taken on wires bent at an angle of about 45 degrees. The swab should be taken by a specialist so that contaminating organisms from the mouth may be reduced to a minimum. Effective results depend upon taking swabs at or very near the laboratory so that they may be planted on to warm plates and immediately placed in the incubator.

As soon as the swabs are taken the mucus is deposited at the edge of a sheep serum-dextrose agar plate, the medium being made according to the following formula:

Ordinary 3 per cent veal agar containing 1 per cent peptone and 0.5 per cent sodium chloride.
Dextrose, 1 per cent.
Adjust reaction carefully to 0.4 acid to phenolphthalein (cold titration) P_H 7.4.

To 500 c.c. of perfectly clear agar add 100 c.c. of sheep serum water made by mixing 1 part of perfectly clear fresh sheep serum with 3 parts of distilled water. Sterilize in the autoclave 40 minutes at 15 pounds pressure.

After depositing the mucus on a plate, a bent sterile glass rod is used to spread it. If the culture is properly taken one plate is sufficient but it is better to use two. Plates should be placed in the incubator at once.

At the end of 24 hours' incubation the plates are looked over and suspicious transparent pearly gray colonies with regular margins are marked, smears are made and stained by Gram's method. Colonies showing a Gram negative diplococcus are planted onto carbohydrate sheep serum agar slants, prepared as follows:

3 per cent sheep serum agar, as the foregoing.
Andrade's indicator 1 c.c. per 100 c.c. medium.
1 per cent carbohydrate, using the following: glucose, maltose, saccharose, levulose, galactose.

It may be advisable at times to plant from the colony onto egg medium slants, and from this transplant onto the carbohydrates. The egg medium is made as follows:

Eggs are washed thoroughly with soap and water. Break the shell and stir yolk and whtie very thoroughly. Strain through sterile cheesecloth and distribute by means of sterile funnels into small test tubes. Heat slowly to 70° C. to expel the air. Solidify at a temperature of 75° C. Sterilize on 2 consecutive days in Arnold steamer at a temperature of 90–100° C.

Those Gram negative diplococci fermenting only maltose and dextrose are agglutinated with a known antimeningococcic serum in dilutions of 1:100 and 1:200.

XI. Plague

If a bubo is present, make smears and cultures from material drawn from a bubo, with a hypodermic syringe; if pneumonic plague, from the sputum, and if septicaemic plague, from the blood.

Smears are stained with carbol thionin or dilute fuchsin, and show typical coccobacilli with the characteristic bipolar staining.

Tubes of plain agar, 3 per cent salt agar, and tubes of broth, with a few drops of oil on the surface, are inoculated with the suspected material. Smears from the growth on plain agar show small typically slender rods—sometimes but not always, showing typical bipolar staining. Smears from salt agar show the 'involution forms,' while in the broth culture is found the characteristic stalactite growth, hanging from the undersurface of the liquid petrolatum.

Two guinea pigs are always inoculated—one by smearing the material on the closely shaven skin of the animal and a second one subcutaneously by pocket inoculation. Inoculated animals are housed in flea-proof containers and are closely watched for a period of 15 days.

All animals, whether dying or killed within 15 days, are posted and the diagnosis confirmed by the macroscopic lesions, such as buboes, enlarged spleen, nodular spleen, or hemorrhagic conditions, as well as by the microscopic examination of the spleen and heart blood and by cultures.

XII. Pneumonia—Type Diagnosis

The culture method used in this laboratory is that of Avery with a modification suggested by Dr. K. F. Meyer, consulting bacteriologist of the bureau.

Several kernels are selected from the specimen of sputum; these are washed four times in sterile saline and are then ground in a sterile mortar with sterile sand. After the sputum is well broken up, about 1 c.c. of broth is added and after mixing thoroughly the supernatant fluid is drawn off with a capillary pipette. This is discharged into a tube of glucose blood broth and incubated for 5 hours in the 37° C. water bath or in the incubator over night. At the end of this time smears are made and stained with Gram's and Hiss' capsule stains. If a good many organisms are present the specimen is centrifuged at low speed (if the red blood cells have settled out during incubation this is not necessary). The clear fluid is pipetted off and centrifuged at high speed; the supernatant fluid is drawn off and the sediment resuspended in sterile saline until the resulting suspension is turbid

Agglutinate this antigen with known Types I, II, and III, pneumococcic antisera using 0.3 c.c. of undiluted serum and 0.3 c.c. of the

antigen. Incubate in the water bath at 37° C. for 1 hour. Put in the ice box over night. Before reading the results in the morning, allow the tubes to stand at room temperature for about 30 minutes.

If cross-agglutination occurs 0.5 c.c. amounts of the antigen, or suspension of suspected pneumococci, are set up with 0.5 c.c. amounts of the following dilutions of the antipneumococcic sera, Serum I, 1 to 20, Serum II, undiluted, Serum II, 1 to 20, and Serum III, 1 to 5.

When setting up the agglutinations make a bile control by adding 0.1 c.c. of bile to 0.5 c.c. of broth culture. Plate out after 20 minutes on blood agar to see if the organisms present are bile soluble.

It is well to plate out from the original specimen or from enrichment medium (glucose blood broth) on blood agar. Pick suspicious colonies after 24 hours' duration. Identify by Gram's and Hiss' capsule stains.

The foregoing culture method of identifying the pneumococcus should be confirmed by mouse inoculation when possible.

XIII. Rabies

Heads of suspected animals are opened and the hippocampus and a part of the cerebellum are removed. A convenient method of opening dogs' heads is to fix the head to a heavy 2-inch plank, on the work table, by driving a large nail through the snout as far back as the length of the nail will permit. A medium incision to the bone is made and the skin including all the tissue down to the bone laid back on either side, exposing the top of the cranium. With a saw or a chisel and hammer make a transverse cut through the bone, avoiding injury to the brain, running the line just posterior to the frontal sinuses. Make two lateral cuts in the same manner, one on either side of the median line converging toward the foramen magnum, from the outer ends of the first incision. With the chisel the piece of bone is pried up from the front edge and laid back. The dura is dissected from the surface of the brain and two lateral incisions made from the middle line, extending outward and backward, through the two hemispheres to the lateral sinus. The hippocampi will be seen as cylindrical white objects lying in depths of the incisions into the ventricles, one on either side.

A small piece of the gray matter from the center of the hippocampus, taken out by cross-sectioning the same, is pressed out between two slides. These smears are allowed to air dry, and are fixed by heat and stained with a modification of William's stain:

Saturated alcoholic methylene blue	2 c.c.
Distilled water	20 c.c.
Saturated alcoholic fuchsin	3–5 drops

The slides are covered with this stain, warmed slightly over a small flame, and then washed and dried.

Microscopical examinations are made for the presence of Negri bodies. These are magenta colored, sharply outlined, regular bodies, containing blue granules. It is always preferable to find intracellular Negri bodies if possible before giving a positive diagnosis. (At least 6 to 8 slides or more are examined before a negative report is given.)

The amount of fuchsin necessary to give the right color to the Negri bodies and the right contrast between them and the ground substance, varies with different brains and should be arrived at by staining and examining a slide after each addition of the dye. This stain should be made up fresh each day.

Negative specimens are no longer inoculated into animals as the length of time for the development of symptoms is too great to allow a person who has been bitten to wait for treatment. Therefore all persons who have been bitten by animals are advised, in case Negri bodies are not found, that the question of treatment must be decided according to the symptoms of the animal at the time of biting.

XIV. Rocky Mountain Spotted Fever

Blood from a suspected case is drawn from a vein, immediately added to a 10 per cent sodium citrate solution in the proportion of 9 to 1, and thoroughly mixed, 1 to 5 c.c. of the citrated blood are inoculated, intraperitoneally, into male guinea pigs. The temperature of the guinea pigs is taken before inoculation and twice daily afterward. A positive report is given if the inoculated animal shows the characteristic temperature curve and scrotal swelling and hemorrhage.

XV. Syphilis

KOLMER'S COMPLEMENT FIXATION TEST IS THE OFFICIAL METHOD OF THE HYGIENIC LABORATORY

Materials Required.—

Glassware and equipment.—One c.c. pipette, graduated into 0.1 c.c. Five and 10 c.c. pipettes graduated into 0.5 c.c.

Test tubes.—Rounded bottom, no lips, measuring 85 m.m. in length with an internal diameter of 12 to 14 m.m. For mixing large volumes of reagents, volumetric flasks are to be preferred to graduated cylinders as insuring greater accuracy. For measuring quantities less than 50 c.c. use a 10 c.c. pipette.

Test tube racks.—Each test requires 6 tubes. Racks carrying 12 rows of 6 tubes each are convenient.

Water bath.—Two water baths of such size as to accommodate all the tests to be run in one day, preferably equipped with automatic electric heating units.

Refrigerator of sufficient ice capacity to maintain a uniform temperature of 6° to 8° C.

Saline solution.—Weigh out 8.5 grams of Merck's Highest Purity sodium chloride and dissolve in neutral 1000 c.c. of distilled water in a chemically clean and dry flask furnished with a gauze-covered cotton plug. Place in an Arnold sterilizer for 1 hour or autoclave for 30 minutes. Chemically pure salt should not be used as interference with hemolysis may result from the unfavorable hydrogen ion concentration of the solution.

CORPUSCLES

Two per cent suspension of freshly collected and washed sheep corpuscles; dose 0.5 c.c. The cells should be washed 4 times, using 4 to 5 volumes of salt solution to one of cells and the last centrifuging should be twice as long as necessary to throw down the corpuscles in the previous washing. This is to evenly pack the washed cells and the speed and duration of centrifuging should be uniform from day to day and based upon experience with the particular centrifuge in use. The volume of corpuscles is read before pouring off the supernatant fluid, after which the cells are added to sufficient saline to make a 2 per cent suspension. A suspension prepared for hemolysin and complement titrations may be kept in the refrigerator until the next day for the main complement fixation tests.

HEMOLYSIN

Antisheep hemolysin diluted with saline and titrated daily with 0.3 c.c. of 1:30 guinea pig complement and 0.5 c.c. of 2 per cent sheep corpuscles. Rabbits may be immunized by intravenous injections of 1 c.c. of 50 per cent suspension of thoroughly washed sheep corpuscles every 3 days for 3 injections, then after an interval of 5 days repeat the series. Bleed and test on the second day after the sixth injection.

COMPLEMENT

Make 1:30 dilution of the mixed sera of 3 or more healthy guinea pigs. If the pigs are to be bled to death, they may be stunned by a blow at the base of the skull and bled from the heart with a 10 c.c. Luer syringe, or the vessels of the neck severed with a sharp pair of scissors and the blood collected in a beaker. If they are to be preserved for future use they should be anaesthetized with ether and bled from the heart. The serum should be allowed to stand on the clot for several hours before centrifuging.

Preservation of complement.—To each c.c. of the clear serum add 0.3 gram pure sodium chloride, dissolve and keep in dark glass bottle immersed in water and cracked ice (1° C.) in the refrigerator. For

use dilute 1 c.c. with 29 c.c. distilled water which gives a 1:30 dilution in 1 per cent saline.

Preserved complements first deteriorate by loss of sensitiveness to fixation by syphilis antibody and antigen (Kolmer).

TITRATION OF HEMOLYSIN AND COMPLEMENT

Titration of hemolysin.

(1) Arrange a series of 10 test tubes and place 0.5 c.c. of varying dilutions of hemolysin in each tube, respectively.

(2) Ordinarily a range of dilutions from 1:5000 to 1:18000 is sufficient but, depending upon the hemolytic activity of the complement and resistance of the corpuscles, higher or lower dilutions may be required. A 1:100 dilution preserved with 0.2 per cent phenol against bacterial contamination may be prepared as follows and kept in a refrigerator for several weeks, from which the higher dilutions are prepared as needed:

Equal parts hemolytic serum and glycerin	2 c.c.
Physiological saline	94 c.c.
5 per cent phenol in saline or water	4 c.c.

(3) The dilutions are prepared as follows in a separate set of large test tubes:

0.2 c.c. of 1:100 + 1.8 c.c. saline = 1:1000
0.2 c.c. of 1:100 + 3.8 c.c. saline = 1:2000
0.2 c.c. of 1:100 + 5.8 c.c. saline = 1:3000
0.2 c.c. of 1:100 + 7.8 c.c. saline = 1:4000
0.2 c.c. of 1:100 + 9.8 c.c. saline = 1:5000
1 c.c. of 1:3000 + 1 c.c. saline = 1:6000
1 c.c. of 1:4000 + 1 c.c. saline = 1:8000
1 c.c. of 1:5000 + 1 c.c. saline = 1:10000
Mix contents of each tube very thoroughly.

(4) Use 0.5 c.c. of each dilution in regulation test tubes and to each add 0.3 c.c. of 1:30 dilution of the same complement and 0.5 c.c. of a 2 per cent suspension of the same corpuscles as used in complement-fixation tests; add 1.7 c.c. saline to each tube to make the total volume 3 c.c. in each.

(5) Mix the contents of each tube and place in the water bath at 38° C. for 1 hour; the unit is the highest dilution of hemolysin showing just complete hemolysis. Two units are employed in the titration of complement and antigen and in the complement-fixation tests.

The following table shows the *ensemble* and results of a titration.

Tube	Hemolysin	Complement 1:30	Corpuscles (2 per cent)	Saline	After water-bath incubation for one hour
1	0.5 c.c. 1:1,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	Complete hemolysis
2	0.5 c.c. 1:2,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	Complete hemolysis
3	0.5 c.c. 1:3,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	Complete hemolysis
4	0.5 c.c. 1:4,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	Complete hemolysis
5	0.5 c.c. 1:5,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	Complete hemolysis
6	0.5 c.c. 1:6,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	Complete hemolysis; unit
7	0.5 c.c. 1:8,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	Marked hemolysis
8	0.5 c.c. 1:10,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	Marked hemolysis
9	0.5 c.c. 1:12,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	Slight hemolysis
10	0.5 c.c. 1:16,000	0.3 c.c.	0.5 c.c.	1.7 c.c.	No hemolysis

In the foregoing titration the unit was 0.5 c.c. of 1:6000; 2 units were contained in 0.5 c.c. of 1:3000.

(6) Sufficient hemolysin is now prepared for the complement titration and complement-fixation tests so that two units are contained in 0.5 c.c.

Titration of complement.—

(1) Arrange 10 test tubes and place 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, and 0.5 c.c. of 1:30 complement in each, respectively; the tenth tube serves as a corpuscle control for this and the hemolysin titration.

(2) Into each of the first 9 tubes place 10 units of antigen so diluted that this amount is contained in 0.5 c.c.

(3) Place sufficient saline solution in each tube to make the total volume about 2 c.c.

(4) Mix contents of each tube and place in a water bath at 38° C. for 1 hour.

(5) Add 0.5 c.c. hemolysin (2 units) and 0.5 c.c. corpuscle suspension (2 per cent) to each tube; mix and reincubate 1 hour.

(6) Ordinarily the smallest amount of 1:30 complement giving complete hemolysis is taken as the unit, but *experience has shown that with the method of primary incubation employed in the complement-fixation tests, namely, from 15 to 18 hours at from 6 to 8° C., this is insufficient; in this test the unit is taken as the amount of complement in the next higher tube.* For example, if hemolysis is just complete with 0.25 c.c. the unit is taken as 0.3 c.c. and double this amount used for the antigen titrations and complement-fixation tests. For convenience this is designated as *two full units of complement.*[1]

[1] Experience has shown that in this test the reactions are unsatisfactory if less than 0.4 c.c. of 1:30 complement is employed; occasionally hyperactive sera yield a unit with 0.1 c.c. of 1:30, but when this occurs it is necessary to use 0.4 c.c. for the dose of complement.

The following table shows the ensemble, the results of a titration, and the method of reading.

TITRATION OF COMPLEMENT

Tube	Complement 1:30	Antigen (10 units)	Saline	Water bath 38° C. for 1 hour.	Hemolysin (2 units)	Corpuscles (2%)	After water bath incubation for one hour
1	0.1 c.c.	0.5 c.c.	1.4 c.c.		0.5 c.c.	0.5 c.c.	No hemolysis
2	0.15 c.c.	0.5 c.c.	1.4 c.c.		0.5 c.c.	0.5 c.c.	Slight hemolysis
3	0.2 c.c.	0.5 c.c.	1.3 c.c.		0.5 c.c.	0.5 c.c.	Marked hemolysis
4	0.25 c.c.	0.5 c.c.	1.3 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis; the exact unit
5	0.3 c.c.	0.5 c.c.	1.2 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis; the full unit
6	0.35 c.c.	0.5 c.c.	1.2 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
7	0.4 c.c.	0.5 c.c.	1.1 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
8	0.45 c.c.	0.5 c.c.	1.1 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
9	0.5 c.c.	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
10	.....	.....	2.0 c.c.		0.5 c.c.	0.5 c.c.	No hemolysis

In practice the hemolysin titration may be placed in the water bath at the same time as the complement titration; at the end of the first incubation of the complement titration the unit of hemolysin is available and 2 units added to all tubes of the complement titration, etc.

(7) *Each 2 full units of complement are diluted with sufficient saline to make 1 c.c., called the dose of complement;* for example if 0.3 c.c. of 1:30 complement is the full unit the dose is 0.6 c.c.

A convenient scheme for diluting the dose of complement to 1 c.c. is as follows: divide 30 by the dose = the dilution to employ in dose of 1 c.c. For example:

Two full units ..... = 0.6 c.c. of 1:30 dilution
Full unit ..... = 0.3 c.c. of 1:30 dilution
Exact unit ..... = 0.25 c.c. of 1:30 dilution

$$\frac{30}{0.6} = 1:50$$

To provide 75 doses of complement (sufficient for testing 12 sera or spinal fluids) would require 75 c.c. of 1:50, prepared by diluting 1.5 c.c. guinea pig serum with 73.5 c.c. of saline solution. This example illustrates the method.

TITRATION OF ANTIGEN

Kolmer antigen is a cholesterinized heart extract prepared by a special process (see Kolmer, *Am. Jour. Syph.*, 1922, 6, 74). This antigen is prepared and distributed by the State Hygienic Laboratory.

HEMOLYTIC AND ANTICOMPLEMENTARY TITRATION

In a series of 10 test tubes prepare the following dilutions of antigen:

1.0 c.c. antigen to 3.0 c.c. saline = 1:4
0.5 c.c. antigen to 2.0 c.c. saline = 1:5
0.5 c.c. antigen to 2:5 c.c. saline = 1:6
1.0 c.c. antigen 1:4 to 1.0 c.c. saline = 1:8
1.0 c.c. antigen 1:5 to 1.0 c.c. saline = 1:10
1.0 c.c. antigen 1:6 to 1.0 c.c. saline = 1:12
1.0 c.c. antigen 1:8 to 1.0 c.c. saline = 1:16
1.0 c.c. antigen 1:10 to 1.0 c.c. saline = 1:20
1.0 c.c. antigen 1:12 to 1.0 c.c. saline = 1.24
1.0 c.c. antigen 1:16 to 1.0 c.c. saline = 1:32

Hemolytic titration.—

(1) In a series of 10 regulation test tubes place 0.5 c.c. of the foregoing dilutions of antigen, respectively.

(2) To each tube add 0.5 c.c. of a 1:10 dilution of normal human serum previously heated for 15 minutes at 55° C. and 1.5 c.c. of saline solution.

(3) Mix the contents of each tube and place in a refrigerator at 6–8° C. for from 15 to 18 hours.

(4) Add 0.5 c.c. of 2 per cent corpuscles suspension to each tube; mix and place in a water bath at 38° C. for 1 hour.

(5) Allow tubes to stand several hours in a refrigerator and read the results: *the smallest amount of antigen just beginning to produce hemolysis is the hemolytic unit.*

The following table shows the *ensemble*, the results of a titration, and the method of reading.

HEMOLYTIC TITRATION OF ANTIGEN

Tube	Antigen 0.5 c.c.	Heated human serum* 1:10	Saline	Refrigerator 6–8° *P.* for 15–18 hours.	Corpuscles (2½)	Water bath 1 hour
1	1:4	0.5 c.c.	1.5 c.c.		0.5 c.c.	Marked hemolysis
2	1:5	0.5 c.c.	1.5 c.c.		0.5 c.c.	Slight hemolysis; unit
3	1:6	0.5 c.c.	1.5 c.c.		0.5 c.c.	No hemolysis
4	1:8	0.5 c.c.	1.5 c.c.		0.5 c.c.	No hemolysis
5	1:10	0.5 c.c.	1.5 c.c.		0.5 c.c.	No hemolysis
6	1:12	0.5 c.c.	1.5 c.c.		0.5 c.c.	No hemolysis
7	1:16	0.5 c.c.	1.5 c.c.		0.5 c.c.	No hemolysis
8	1:20	0.5 c.c.	1.5 c.c.		0.5 c.c.	No hemolysis
9	1:24	0.5 c.c.	1.5 c.c.		0.5 c.c.	No hemolysis
10	1:32	0.5 c.c.	1.5 c.c.		0.5 c.c.	No hemolysis

*May be omitted, in which case 2 c.c. saline are added to each tube instead of 1.5 c.c.

Anticomplementary titration.—

(1) In the first 10 tubes of a second series of 12 tubes place 0.5 c.c. of the foregoing dilutions of antigen, respectively.

(2) To each of the first 11 tubes add 0.5 c.c. of a 1:10 dilution of *normal* human serum previously heated at 55° C. for 15 minutes.

(3) Add 1 c.c. of diluted complement (carrying 2 full units) to each of the 12 tubes.

(4) To the eleventh tube add 0.5 c.c. and to the twelfth tube 1 c.c. saline solution.

(5) Mix all tubes and place in a refrigerator at 6 to 8° C. for 15 to 18 hours.

(6) Place tubes in a water bath at 38° C. for 5 to 10 minutes (not longer) and then add 0.5 c.c. hemolysin (2 units) and 0.5 c.c. of the 2 per cent suspension of corpuscles to each tube; mix and place in a water bath at 38° C. for 1 hour. Place the tubes in a refrigerator for a few hours and read the results.

(7) The *anticomplementary* unit is the smallest amount of antigen producing some inhibition of hemolysis. The eleventh tube is the serum control; the twelfth tube is the hemolytic system control, and both should show complete hemolysis.

The following table shows the *ensemble,* the results of a titration, and method of reading:

ANTICOMPLEMENTARY TITRATION OF ANTIGEN

Tube	Antigen	Heated human serum* 1:10	Complement (2 full units)	Refrigerator 6–8° C. for 15–18 hrs. followed by water bath 5 to 10 minutes.	Hemolysin (2 units)	Corpuscles (2½)	Water bath 1 hour
1	1:4	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Slight inhibition of hemolysis
2	1:5	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Slight inhibition of hemolysis (unit)
3	1:6	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
4	1:8	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
5	1:10	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
6	1:12	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
7	1:16	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
8	1:20	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
9	1:24	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
10	1:32	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
11	0.5 saline	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis
12	1.0 saline	.....	1.0 c.c.		0.5 c.c.	0.5 c.c.	Complete hemolysis

*May be omitted and 0.5 c.c. saline added instead.

THE QUANTITATIVE COMPLEMENT-FIXATION TEST

(1) Sera should be properly separated from the clot and heated in a water bath at 55° C. for 15 minutes; spinal fluids are used unheated. The tests should be set up in the following order: serum first, followed by antigen, and lastly, by complement.

(2) For each *serum* arrange 6 regulation test tubes and place saline solution in the following amounts respectively: 1.2, 0.5, 0.5, 2.0, 0.5, and 0.5. (*a*) Into the first tube place 0.3 c.c. of the inactivated serum and mix by drawing up in pipette and expelling at least 3 times. Transfer 0.5 c.c. to tube 2 and tube 6. (*b*) Mix contents of tube 2 and transfer 0.5 c.c. to tube 3. (*c*) Mix contents of tube 3 and transfer 0.5 c.c. to tube 4. (*d*) Mix contents of tube 4, discard 1.5 c.c., and transfer 0.5 c.c. to tube 5. (*e*) Mix contents of tube 5 and discard 0.5 c.c.

Each tube now contains 0.5 c.c. carrying 0.1, 0.05, 0.025, 0.005, 0.0025, and 0.1 c.c. (serum control).

(3) For each *spinal fluid* arrange 6 regulation tubes and place 0.5 c.c. saline in tubes 2, 3, 4, 5, and 6.

Into the first, second, and sixth tubes place 0.5 c.c. spinal fluid; mix contents of tube 2 and transfer 0.5 c.c. to tube 3; mix contents of tube 3 and transfer 0.5 c.c. to tube 4. Mix contents of tube 4 and transfer 0.5 c.c. to tube 5. Mix contents of tube 5 and discard 0.5 c.c. Each tube now contains 0.5 c.c. carrying 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.5 c.c. (control).

(4) To the first 5 tubes of each set, add 0.5 c.c. antigen dilution (carrying 10 antigenic units).

(5) After an interval of 5 to 30 minutes add 1.0 c.c. of complement (2 full units) to each tube.

(6) Include the following controls: (*a*) *antigen control* tube carrying 0.5 c.c. of the diluted antigen, 1 c.c. of the diluted complement, and 0.5 c.c. of saline solution; (*b*) *hemolytic system control* carrying 1 c.c. of the diluted complement and 1 c.c. of saline solution; (*c*) *corpuscle control* carrying 2 c.c. of saline solution; (*d*) *positive* and *negative* serum controls should be included using syphilitic and normal sera, respectively, set up in the various amounts as described in the foregoing.

(7) Start the preparation of a reading scale as follows: (*a*) Heat 6 c.c. of the diluted complement in a water bath at 55° C. for 15 minutes. (*b*) Prepare a solution of hemoglobin by dissolving 2 c.c. of the 2 per cent corpuscle suspension in 4 c.c. of plain water. (*c*) Arrange a series of 5 regulation test tubes numbered 1 to 5 and place

in each: 0.5 c.c. of the diluted antigen and 1 c.c. of the heated diluted complement. (*d*) Add hemoglobin solution to the first 4 tubes as follows: 1.5, 1.13, 0.75, and 0.38 c.c. (*e*) Add 0.24 c.c. physiologic saline solution to No. 2; 0.5 c.c. to No. 3; 0.74 c.c. to No. 4, and 1 cc. to No. 5. Corpuscles are added the following day.

(8) Mix the contents of all tubes gently but thoroughly and place the tubes in a refrigerator at 6 to 8° C. for from 15 to 18 hours.

(9) Warm the tubes in a water bath at 38° C. for from *5 to 15 minutes but not longer*[2] and add 0.5 c.c. hemolysin (carrying 2 units) to all tubes except the tubes of the corpuscle control and the reading scale; thoroughly mix the 2 per cent corpuscle suspension (which has been carried over in a refrigerator from the previous day) and add 0.5 c.c. of it to all tubes except those of the reading scale.

(10) Add corpuscle suspension to the tubes of the reading scale as follows: 0.13 c.c. to tube 2; 0.25 c.c. to tube 3; 0.38 c.c. to tube 4, and 0.5 c.c. to tube 5.

(11) Mix the contents of all tubes gently but thoroughly and place in a water bath at 38° C. for 1 hour (the water must reach above the level of the contents in the tubes).

(12) Place all tubes in a refrigerator for 1–3 hours to permit the partial settling of non-hemolyzed corpuscles; the degree of inhibition hemolysis is then read off and recorded for each tube with the aid of the reading scale as, + (1), ++ (2), +++ (3), or ++++ (4). All serum, antigen, and hemolytic controls should show complete hemolysis.

(13) Tube 1 of the color scale shows a — reaction; tube 2 shows a +; tube 3 shows a ++; tube 4 shows a +++, and tube 5 shows a ++++ reaction.

Experience has shown that the reactions may be interpreted as follows:

Very strongly positive if there is partial or complete fixation of complement in the first 4 of all 5 of the fixation series.[3] Examples: 4431—; 44421; 1442—.

Strongly positive if there is partial or complete fixation of complement in the first 3 tubes. Examples: 441 — —; 421 — —; 241 — —.

---

[2] This preliminary warning may be omitted; if continued for more than 15 minutes, incomplete hemolysis may occur.

[3] Occasionally a serum will show less fixation of complement in the first tube carrying 0.1 c.c. serum than in the second tube carrying 0.02 c.c. as a ++, ++++, +++, +, — and — (control) reaction (recorded as 2, 4, 3, 1, —). It may be assumed that this is caused by the presence of natural antisheep hemolysin; but the phenomenon occurs with hemolysin-free sera and it is believed by Kolmer that it is due to the presence of other serum constituents in this relatively large amount of serum which interferes with the fixation of complement by antigen and syphilis antibody.

Moderately positive if there is partial or complete fixation of complement in the first 2 tubes. Examples: 44 — — —; 41 — — —; 31 — — —.

Weakly positive if there is partial or complete fixation of complement only in the first tube. Examples: 4 — — — —; 3 — — — —; 1 — — — —.

Negative when all tubes show complete hemolysis. (— — — — —.)

The following reactions with a syphilitic serum and spinal fluid show the method of recording and reporting:

Serum 0.1	c.c. = ++++
Serum 0.02	c.c. = ++++
Serum 0.004	c.c. = ++
Serum 0.002	c.c. = +
Serum 0.001	c.c. = —
Serum 0.1 (control)	c.c. = —
Spinal fluid 0.5	c.c. = ++++
Spinal fluid 0.25	c.c. = ++++
Spinal fluid 0.125	c.c. = ++++
Spinal fluid 0.0625	c.c. = ++++
Spinal fluid 0.03125	c.c. = ++
Spinal fluid 0.5 (control)	c.c. = —

XVI. Tuberculosis

All specimens of sputum are autoclaved before examining. This process breaks up the mucus and renders the specimens safe to handle.

Thick smears are made on new slides and after fixing by heat are stained in jars, at least 10 minutes, with the acid fast stain by the cold method. The smears are washed in water until all surplus carbol fuchsin is removed and stained with Gabbett's stain (in jars), from 30 seconds to 1 minute, depending on the thickness of the smears.

The non-acid fast organisms take the stain very lightly so that the tubercle bacilli appear a very dark red against a faint blue background.

Acid fast stain:

Fuchsin basic	4.0 gms.
Phenol crystals	8.0 gms.
Distilled water	150.0 c.c.

Gabbett's stain:

Sulphuric acid 25%	100.0 c.c.
Methylene blue	1.0 gm.

The Gabbett's stain should be changed frequently.

XVII. Typhoid Fever

Blood culture—

About 5 c.c. of the patient's blood are added to 250 c.c. of broth and incubated at 37° C. for 24 hours; smears from the blood culture are stained by Gram's method and if no contaminating organisms are found, several loopfuls are planted on an agar slant. After 24 hours' incubation, the culture is washed down with sterile saline and is agglutinated with a typhoid-agglutinating serum of known titer.

If the blood culture is found to be contaminated, it must be planted on Endo medium, suspicious colonies fished out and planted into Russell's double sugar medium; if the fermentation reactions are satisfactory for typhoid, the organism is agglutinated with a known serum, as described above.

The mailing containers furnished for sending blood specimens to the laboratory contain two bottles: one small one equipped with a lancet in the cork for puncturing the lobe of the ear or the finger, for the Widal test, and a larger one for blood culture. The printed slip accompanying this outfit reads as follows:

> This mailing case contains bottles for submitting samples of blood for *both* the Widal and the blood culture test for typhoid fever. The blood culture yields results much earlier than the Widal and is absolutely conclusive when positive.
>
> The large vial is for the blood culture. It contains sterile ox bile, which prevents clotting of the blood and which preserves the vitality of the typhoid bacillus until it reaches the laboratory.
>
> *Directions:* Take blood from a vein as for the Wassermann test and add it to the bile in the bottle until it is three-quarters full. Cork tightly and return to the laboratory.
>
> It is advisable to send in both tests; the small vial half-full of blood from ear or vein and the large vial with blood from vein mixed with bile as above described.
>
> Please fill out blank form carefully, using a good pencil and firm pressure so as to secure a legible carbon copy.

The bottle containing blood and bile is placed in the incubator for a few hours then plated out on Endo and eosin, methylene blue media. Suspicious colonies are identified as described under examination of excreta.

The agglutination test—

The microscopic method is approved provided fresh fluid blood is used instead of dried specimens, and that some method of making accurate dilutions is used. The preferred method, however, is the macroscopic test. The technic used for the macroscopic test in the Hygienic Laboratory is as follows:

The patient's blood (taken from ear or vein in sufficient amount to yield 0.1 c.c. of serum) is centrifuged. Eight small test tubes are set up in a rack and into the first is placed 1.9 c.c. and in each of the others 1.0 c.c. of normal salt solution. To the first tube add 0.1 c.c. of patient's serum. Mix the contents and carry over 1 c.c. to the second tube. The contents are mixed and 1 c.c. carried to the third tube, and so on until the seventh tube has been finished, at which point 1 c.c. of the mixture is thrown away instead of being placed in the eighth tube. You now have 1 c.c. of serum dilution in each tube ranging from 1–20 in the first, to 1–1280 in the seventh, with plain normal salt solution in the eighth tube. The eighth tube serves as a control against spontaneous agglutination. Next 0.1 c.c. of the killed (formalinized) typhoid antigen is added to each tube, including the control, the rack is shaken, placed in the 56° C. bath for 2 hours, then set aside at room or ice box temperature for a few hourss before reading results, which is best done by holding the tubes up against a light background. In lieu of the 56° C. bath the racks may be placed in the incubator at 37° C. over-night and read in the morning. A short cut, which has proved safe in our hands, is to place the tubes in the 56° C. bath for half an hour, then centrifuge them at high speed for a few minutes. A sharp tap on the side of the tube near the bottom will cause the sediment to rise from the bottom and it is easy to distinguish flocculation from mere sedimentation of the bacilli. In the latter case the packed bacilli rise in a characteristic 'swirl' which will not be confused with the coarser particles that rise uniformly through the clear supernatant fluid in those tubes showing complete agglutination.

Any laboratory wishing to use the State Hygienic Laboratory standard antigen will be sent a supply on request. It will keep for several months in an ice box.

Excreta—

Whenever possible the fresh specimen is used, but when specimens are sent to the laboratory by mail a special outfit is required which contains a 30 per cent solution of glycerin in saline. It is requested that the soft stool be obtained, preferably the second after taking a cathartic. Calomel should not be taken. A small portion, about the size of a bean, of a well-mixed and broken specimen is transferred to a tube of nutrient broth; 0.1 c.c. of this mixture is transferred to a second tube and 0.1 c.c. from the second to a third. These dilutions are allowed to stand about 10 or 15 minutes before plating.

One or two drops of the broth mixtures are well spread, using a bent glass rod, over the surface of freshly prepared Endo and eosin methylene blue plates. Use 1 plate for each broth dilution. After 24 hours' incubation the clear, transparent, pearly colonies are seeded by streak and stab inoculation into Russell's double sugar agar. In this medium the acid and gas production is obtained, while the slant provides growth for the macroscopical agglutination, hanging drop for motility, and smear for staining by Gram's method. The macroscopic agglutination confirms the cultural findings, and the serum used is one of known titer. Should there be any doubt regarding the sugar reaction in the Russell's medium, fermentation tubes of glucose, lactose, saccharose, and mannit broth should be inoculated as a check.

XVIII. Typhus Fever

Blood from a suspected case is drawn from a vein and immediately added to a 10 per cent sodium citrate solution in the proportion of 9 to 1 and thoroughly mixed.

At the laboratory guinea pigs are inoculated intraperitoneally with from 2 to 5 c.c. of the citrated blood. The temperature of the guinea pigs is taken before inoculation and then twice daily. Laboratory findings depend upon the temperature chart, a positive report being given when the characteristic rise takes place about 7 to 10 days after inoculation.

Weil-Felix agglutination test.—A culture of Proteus X_{19} is kept on agar, and transplanted once a month.

A salt solution suspension of the living culture is made of a density that, when 0.1 c.c. is added to 1.0 c.c. of fluid, a barely perceptible turbidity results. This is used as the antigen in an agglutination test with the patient's serum, carried out exactly as described for typhoid fever.

XIX. An Outline of the Types of Specimens Required and the Procedures Usually Employed in the Laboratory Diagnosis of the Reportable and Quarantinable Diseases.

Anthrax. *Clinical specimens:* Pustule content—microscopic, cultural and pathogenicity tests.

Epidemiological specimens: Blood specimens, spleen (preserved on filter paper), a whole ear or a piece of hide of cattle, sheep, horses, etc. Sputum (wool-sorters' disease)—examination by microscopic, cultural, and pathogenicity tests.

BERI-BERI. Clinical diagnosis.

BOTULISM. *Clinical specimens: Stool specimens*—enrichment cultures —toxicity test.

Necropsy specimens: Intestines, spleen and liver-enrichment cultures—toxicity tests—toxin typing with antitoxin.

Epidemiological specimens: Suspected food (usually canned)—centrifuged and filtered—toxicity test on guinea pigs by feeding or subcutaneous inoculations on mice—typing of the toxin with antitoxins; cultures.

CEREBROSPINAL MENINGITIS. (Q)*

Clinical specimens: Spinal fluid—microscopic and cultural tests on serum media at 37° C. Agglutination tests with therapeutic serum.

Blood culture in early cases—serum agglutination tests during convalescence.

Carriers: Nasopharyngeal swabs rolled over warm blood serum agar plates—characteristic colonies of Gram-negative cocci—no growth at room temperature—agglutination test with polyvalent and specific sera.

CHICKEN POX. *Clinical lesions:* In exceptional cases collect vesicle content and scarify cornea of rabbits—unilocular vesicle contains no cell inclusions—vesicle content gives negative skin reactions on rabbits previously vaccinated. Vaccination of previously unvaccinated patient (should take).

CHOLERA. (Q) *Clinical specimens: Stool specimens*—direct on surface of Dieudonné's or Aronson's agar, or enrichment in peptone solution and then plating—microscopic and cultural examination—agglutination tests.

Carriers: Stool or bile specimens (duodenal tube) enriched in alkaline peptone solution—plating on selective (alkaline) media—agglutination. Serum of patients: agglutination tests.

DENGUE. In doubtful cases blood examination (leucopenia) and injection of guinea pigs with fresh whole blood of patients.

---

* Q = Quarantinable.

DIPHTHERIA. (Q) *Specimens of suspected clinical cases:* Swabs from membrane, nasopharynx or wounds—microscopic examination and elective enrichment on Loeffler's or Greenspoon's medium. Microscopic examination. Virulence tests on guinea pigs or rabbits if necessary.

Blood serum: Kellogg's test for presence or absence of antitoxin.

Carriers: Enrichment of nasal, pharyngeal, and aural secretions on serum mediums. Microscopic and cultural study. Virulence tests with whole cultures. Agglutination tests according to Doyle if necessary.

DYSENTERY. *Specimens of clinical cases: Fresh* flake of intestinal bloody mucus or urine collected before the tenth day of the disease plated immediately on eosin, methylene blue agar. Isolation of Gram negative, non-lactose fermening organisms. Slide agglutination with polyvalent serum. Purification—carbohydrate fermentation tests. Agglutination tests with specific sera (5 types).

Repeated stool cultures are important.

Blood serum: Agglutination tests (1:30) with 11 standard formalinized antigens (typhosus, para A and B, Shiga, Flexner, English types V, W, X, Y, and Z, *B. dispar, B. ambigus*).

Necropsy material: Scrapings of the mucous membrane of the ulcerated intestines—plated and studied as clinical specimens.

Carriers: Fresh stool specimens during relapses treated in the same manner as material from acute cases.

Blood serum: Agglutination tests with standard antigens.

ENCEPHALITIS (EPIDEMIC). (Q) Clinical and anatomical (necropsy brain specimens fixed in 10 per cent formaldehyde).

ERYSIPELAS. Tissue fluid or contents of blebs. Identification of hemolytic streptococcus isolated on blood plates by agglutination tests with specific sera.

FLUKES. Routine fecal examinations of carefully selected stool sample (ova of Schistosoma are unevenly distributed). Sputum examinations for lung flukes.

Food Poisoning. *Chemical specimens:* Excreta samples and vomit plated on eosin, methylene blue agar; identification of the Gram negative, non-lactose fermenting organisms by carbohydrate and agglutination reactions.

Blood cultures: Identification of Gram negative organisms by agglutination tests.

Blood serum: Collected 15–30 days after onset. Agglutination tests with 6 standard antigens (*B. enteritidis,* Derby, *B. aertrycke,* Stanley, Newport, *B. suipestifer*).

Epidemiological specimens: The following specimens should be collected and sent packed in ice.

(1) *Any portion of the supposed peccant food* (even small scraps, samples of food of similar origin are usually useless)—isolation of organisms belonging to the Salmonella group either by direct plating or after enrichment in brilliant green broth; demonstration of the toxicity of the food by feeding animals with it or by injecting extracts of the suspected food into animals; demonstration of the production of specific agglutinins in animals through the injection into them of suitable emulsions of the incriminated food.

(2) *Necropsy materials from fatal cases:* The spleen, pieces of liver, piece of small intestines, pieces of large intestines, kidney are the most useful.

German Measles (Rubella). Differential blood count (leucopenia and lymphocytosis confirms diagnosis of German measles. Negative Dick test in recovered cases in absence of scarlet fever history suggests scarlet fever.

Glanders. *Clinical specimens:* Contents of pustules or nodules are examined microscopically and by cultures on potato serum agar. Pathogenicity tests on male guinea pigs.

Blood serum: Agglutination, precipitation and complement-fixation tests.

'Latent' infections in equidae: Hypersensitiveness tests: ophthalmic or intrapalpebral mallein tests. Serum agglutination, complement-fixation and conglutination tests. Bacteriological examination of pathological specimens (nasal discharge, pus of pustules and pulmonary nodules).

Gonococcus Infections.

In *acute stage,* abundant discharge from genito-urinary passages or conjunctiva (in ophthalmia) furnishes specimens for slides or cultures—microscopic (Gram stain) and cultural study.

In *chronic cases* secretions must be obtained in

Male: From the anterior urethra, para-urethral canal, Cowper's glands, behind stricture, prostate or seminal vesicles, exceptionally bladder or kidney.

Female: From vulva in vulvovaginitis of children; in adults: urethra, para-urethral ducts; glands of Bartholin, cervix, uterus and tubes.

Blood serum tests: Complement-fixation test.

Hookworm. Examination of feces by the centrifugation and the brine flotation-loop method.

Influenza. Although the etiology is still uncertain—smears and cultures on chocolate agar inoculated with throat, naso-pharyngeal and bronchial secretions should be examined for *B. influenzae.*

Blood: Differential blood count (leucopenia);

Leprosy. (Q) Biopsy specimens, lymph from non-ulcerated or ulcerated nodules, nasal swabs are used for the preparation of films and the demonstration of acid fast bacilli in characteristic clusters.

Blood: Differential blood count (eosinophila); during febrile attacks lepra bacilli may be found in the leucocytes.

Malaria. (Q) Blood taken during the pyrexia—thin and even films and a carefully dried 'thick drop' of blood on the same slides—the 'thick drop' is hemolysed in tap water before fixation with methyl alcohol—Wright's or Giemsa's stain.

Search for parasites; monocytosis; polychromasia.

Measles. Differential blood count (leucocytes, slight increase of eosinophiles during eruptive stage increase of mononuclear cells). Rabbits may be inoculated with fresh blood.

Mumps. (*Parotitis epidemica.*) Differential blood count (absence of leucocytosis or slight lymphocytosis).

Ophthalmia Neonatorum. *See* gonococcus infection.

Paratyphoid. *See* typhoid fever and food poisoning.

Pellagra. Clinical diagnosis.

PLAGUE. (Q) *Specimens of suspected clinical cases:*

Bubonic: Pustule content or lymph from a carbuncle or lymph-node. Microscopic examination (bipolar stain), cultures on salt agar, broth and milk, pathogenicity tests on guinea pigs (by vaccination) and identification of the cultures by agglutination tests.

Pneumonic: Examination of sputum by the same methods as given above.

Blood: Cultures and agglutination tests (not before the ninth day).

Examination of rats and ground squirrels: Careful autopsies in daylight—microscopic examination 'vaccination' of guinea pigs with tissues. Cultures on gentian-violet agar—identification by cultivation on salt agar, broth and by agglutination tests.

PNEUMONIA. *Clinical specimens:* Sputum—microscopic examination —cultures in blood broth or on blood plates. Typing and identification by precipitation or agglutination tests. Bile solubility test. Enrichment and isolation by mouse inoculations.

Blood: Cultures and differential blood count (leucocytosis).

Urine: Precipitin tests.

Carriers: Sputum; throat and nose swabs enriched in blood broth or by intraperitoneal inoculations of mice.

POLIOMYELITIS. (Q) *Spinal fluid* clear and hazy, normal cell content to slight lymphocytosis—sterile.

Brain and cord tissues of necropsy cases for sections.

Carriers: Nasopharyngeal washings may be tested on monkeys.

RABIES. *Suspected animals:* Keep isolated under careful observation for at least 14 days. Ship the head of the dead animal packed in ice. Examine smears or sections prepared from the hippocampus, cerebellum for Negri bodies.

Doubtful findings require intracerebral inoculation of guinea pigs or rabbits.

ROCKY MOUNTAIN FEVER. *Clinical specimens:* Fresh defibrinated blood may be inoculated intraperitoneally into male guinea pigs—temperature records and testicular lesions support the diagnosis. Differential blood count (moderate mononuclear leucocytosis).

Suspected ticks may be placed on guinea pigs. Note temperatures and lesions.

Scarlet Fever. (Q) *Clinical cases:* Throat swabs are cultured on blood agar—the hemolytic streptococci isolated in pure culture. Cultivated in blood broth for 2 to 4 days—the culture is filtered through a Berkefeld W candle—the presence of a specific toxin is demonstrated by skin tests on a person known to be susceptible to scarlet fever. A portion of the toxin is also mixed with sterile convalescent or concentrated scarlet fever antitoxin. Specific anti-streptococcic rabbit sera or convalescent sera in which the antibodies have been concentrated with ammonium sulfate may be used for the identification of the streptococci. Negative Dick test early in disease or positive Dick test after recovery against scarlet fever diagnosis.

Convalescent cases or carriers: Cultures are prepared from the discharges of the sinuses, glands, middle ears, etc. The procedures outlined above are followed.

Smallpox. Vesicle content may be sacrified on the cornea of a rabbit and the cells of the multilocular vesicle which develops are examined for the presence of Guarnieri vaccine bodies. The content may also be tested on rabbits previously vaccinated with vaccine virus—a definite intradermal reaction indicates smallpox virus. Vaccination of patient in clinically doubtful cases.

Syphilis. *Tissue fluids of primary lesions:* Demonstration of *Treponema pallida* by dark field illumination or stained films (negative staining). (Intratesticular injections of lymph.)

Secondary and tertiary: Wassermann reaction with blood serum and spinal fluid; Kahn precipitin test.

Tetanus. Pus or fragments of tissue examined microscopically and enriched in brain medium or inoculated subcutantously on mice (Pasteur pipette)—toxicity of cultures tested on mice. Toxin identified by toxin-antitoxin neutralization tests.

Purification of cultures is difficult.

Cerebrospinal fluid of clinical cases may be tested for the presence of tetanus toxin by subcutaneous inoculation of mice.

Trachoma. Etiology not definitely established. Inclusion bodies and hemoglobinophilic bacteria have diagnostic significance.

Tuberculosis. *Examination of sputum*—microscopic demonstration of the tubercle bacilli with and without previous concentration. Cultivation by Petroff's method. Cytology of the sputum—inoculation of guinea pigs.

Examination of feces: Suspension of stool treated antiformin—sediment is colored with acid fast stain.

Examination of urine: Concentration by centrifugalization—sediment used for films and guinea pig inoculation.

Examination of spinal fluid: Fibrin coagulatum is used for films and guinea pig inoculation.

Blood: Complement fixation test.

TYPHOID FEVER. (Q) *Suspected clinical cases:*

Blood culture in broth or peptone-ox-bile—subculture on differential media—identification by carbohydrate and agglutination tests.

Blood serum: Agglutination (Widal) test with standardized antigens (*B. typhosus,* paratyphosus A and B).

Stool cultures: Preservation of specimens with glycerin; direct plating or enrichment in bile—brilliant green broth—plating on differential media—identification of the colonies by carbohydrate and agglutination reactions.

Urine: Diazo reaction; enrichment of a sample of a 24-hour specimen in broth and then plating.

Rose spots: Skin sterilized with alcohol and ether; incision, fluid enriched in broth.

Sputum, pus, etc.: Plated directly or after enrichment—identification by agglutination test.

Convalescent case: No case is discharged without 3 consecutive negative stool and urine cultures. Two bile cultures (duodenal tube) should be made in doubtful cases.

Carriers: Blood serum: Widal tests with standard antigens (dilutions 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, etc.).

Stool cultures: Specimens to be collected after the administrations of a mild cathartic (Elaterine); enrichment in brilliant green bile is advantageous. Plating and identification by the usual methods.

Duodenal cultures: Suspicious cases with a Widal of 1:50 or above should be submitted to bile cultures (Einhorn duodenal tube). Bile plate directly or after incubation for 24 hours.

Urine cultures: (On 2 successive days) enrichment of a sample of a 24-hour specimen in broth and then plating.

Typhus Fever. (Q) *Blood:* Weil-Felix reaction: serum agglutination with *B. proteus* X_{19} (fourth-sixth day of the disease positive).

Injection of guinea pigs with fresh blood. Temperature reaction with demonstration of histologic changes in central nervous system; ability of the virus containing brain to produce on inoculation in rabbits specific agglutinins for proteus X_{19}.

Whooping Cough. Sputum during the early stages of the disease (first week)—plated on acid Bordet-Gengou medium—identification of the Gram-negative bacilli by cultivation on glycerin-ascites agar slants and by agglutination tests.

PART VI

OUTLINE OF COURSE IN MEDICAL ZOOLOGY

PREPARED BY

E. L. WALKER

PART VI

OUTLINE OF COURSE IN MEDICAL ZOOLOGY

PREPARED BY

E. L. WALKER

I. Introduction

Definition.—Parasitology in its wider sense includes the study of all organisms that live and obtain their nourishment, in part or as a whole, on or in other living organisms. In its narrower and now generally accepted sense, medical parasitology or medical zoology is concerned only with the *animal* parasites of man and other animals; the vegetal parasites are allotted to the sciences of bacteriology and mycology.

Divisions.—I. Protozoology, which is concerned with the morphology, life-history, and pathogenesis of the unicellular animal parasites.

II. Helminthology, which is concerned with the morphology, life-history, and pathogenesis of the parasitic worms.

III. Medical entomology, which is concerned with the parasitic, and especially with the blood-sucking, insects and arachnids that are medically important chiefly as transmitters of infectious diseases.

IV. General medical zoology, which is concerned with the rôle of the higher animals as vectors or reservoirs of infectious diseases, and with the venomous animals.

Relation to medicine.—Medical zoology bears a relation to tropical medicine that is in some respects similar to that borne by bacteriology to general medicine. The infectious diseases characteristic of warm climates are largely of parasitic etiology; while the infectious diseases of temperate and cold climates are characteristically of bacterial etiology. The relation of parasitology to tropical medicine is, however, broader than that of bacteriology to general medicine; for many of the parasitic and some of the bacterial diseases are transmitted by blood-sucking temporary parasites of the groups Insecta and Arachnida. It should be borne in mind, however, that the parasitic diseases, while characteristically tropical, have in many cases an extra-tropical range and may be met with in any climate. For example, amoebic dysentery, malaria, and hookworm disease are endemic in California.

Methods of medical zoology.—The methods of medical zoology differ in several respects from the methods of its sister science, bacteriology. The bacteria are simple undifferentiated organisms and are, almost without exception, cultivable on artificial media. They are diagnosed primarily by cultural and biochemical characters, and only secondarily by morphological characteristics. On the other hand, the animal parasites are more highly organized and relatively well-differentiated organisms, and in only exceptional cases can they be cultivated. Therefore the animal parasites are identified, not by cultural characteristics, but by morphological characters, usually under the microscope. Moreover, while the bacteria have a simple life-history, multiplying by simple fission and in some cases developing resistant spores, the animal parasites have more or less complex life-histories which are frequently passed in more than one host. It is an important function of medical zoology to elucidate these complex life-histories; for a knowledge of the method of infection and of the effective means of control of parasitic and insect-borne diseases depends upon a complete understanding of the life-histories of the parasites and intermediary hosts involved.

Textbooks:

FANTHAM, STEPHENS AND THEOBALD. *The animal parasites of man.* William Wood & Co., New York, 1916.

HEGNER, CORT AND ROOT. *Outline of medical zoology.* The Macmillan Co., New York, 1923.

NEUMANN AND MAYER. *Atlas und Lehrbuch wichtiger tierischer Parasiten und ihrer Nebenträger.* Munich, 1914.

BRUMP. *Précis de parasitologie.* Masson et Cie., Paris, 1922.

DOFLEIN. *Lehrbuch der Protozoenkunde.* Gustav Fischer, Jena.

MINCHIN. *An introduction to the study of the protozoa.* Edward Arnold, London, 1912.

HEGNER AND TALIAFERRO. *Human protozoology.* The Macmillan Co., New York.

PATTON AND CRAGG. *A text book of medical entomology.* Christian Lit. Soc. for India. London, Madras and Calentia.

HERMS. *Medical and Veterinary entomology.* The Macmillan Co., New York.

MANSON–BAHR. *Manson's tropical diseases.* William Wood & Co., New York, 1921.

CASTELLANI AND CHALMERS. *Tropical medicine.* William Wood & Co., New York, 1919.

STITT. *Diagnostics and treatment of tropical diseases.* P. Blakiston's Sons & Co., Philadelphia.

MENSE. *Handbuch der Tropenkrankheiten.* Leipzig, 1913–.

Periodicals:

Tropical Diseases Bulletin. London, 1912–.

Annals of Tropical Medicine and Parasitology. Liverpool, 1907–.

Parasitology. Cambridge, England, 1908–.

Journal of Parasitology. Urbana, Illinois, 1914–.

Memorias do Instituto Oswaldo Cruz. Rio Janeiro, 1909–.

Indian Journal of Medical Research. Calcutta, 1913–.

Bulletin de la Société de Pathologie Exotique. Paris, 1907–.

Archiv für Schiffs- und Tropen Hygiene. Leipzig, 1897–.

Journal of Tropical Medicine and Hygiene. London, 1898–.

Transactions of the Society of Tropical Medicine and Hygiene. London, 1907–.

American Journal of Tropical Medicine. Baltimore, 1921–.

Philippine Journal of Science. Manila, 1906.–.

II. Methods for Diagnosing and Preserving Animal Parasites

Blood protozoa may be examined living in fresh blood preparations under a cover glass. Stained, permanent preparations are made from thin blood films, air dried, and stained with Wright or Giemsa stain. Examine with oil-immersion objective without use of a cover glass. Since the parasites are often scarce in the blood, careful search of several preparations and repeated examinations are necessary to exclude an infection. The thick blood film preparation assists in the diagnosis of malaria and trypanosomiasis by concentrating the parasites. Several drops of blood are placed together on a microscopic slide and slightly spread to form a thick film. After it is thoroughly dry, the preparation is dehemoglobinized in distilled water, air dried, and stained with Wright or Giemsa stain. The parasites are concentrated but may be more or less deformed in the thick films, and experience is necessary to identify them. The trypanosomes of African sleeping sickness are also diagnosed by the microscopic examination of fresh or stained preparations of the material obtained by puncture of the cervical glands, where the parasites are more numerous than in the blood, and by the inoculation of the blood of the patient into laboratory animals.

The *Leishmaniae* are present only rarely in the circulating blood of the visceral type of disease. Diagnosis is made on dry film preparations, made from scrapings from the cutaneous lesions or from material obtained by spleen or liver puncture of visceral leishmaniasis, stained with Wright or Giemsa stain. The Leishmaniae can be cultivated on the Novy-McNeal-Nicolle medium. (See textbooks for media and methods.)

Intestinal protozoa are usually diagnosed in slide preparations of fresh feces. The fecal specimen should be examined within a short time after it is passed, since the motile, trophozoite stage of intestinal protozoa dies quickly and disintegrates outside of the body. A small bit of the feces is thoroughly mixed with a drop of saline or water and a cover glass placed over the preparation. Too much feces renders the preparation opaque, while too much fluid will allow the cover glass to float about during the application of the oil-immersion objective. Use low magnification for searching the preparation, and high magnification for identifying the parasites. The use of an iodine solution (5 per cent potassium iodide in physiological salt solution, saturated with iodine) for making the fecal preparation, stains the nuclei and glycogen masses of protoboan cysts and aids in their identification.

Satisfactory permanent stained preparations of intestinal protozoa can be made only of fecal smears fixed while moist in Schaudinn's fluid (saturated aqueous solution of mercuric chloride, 2 parts; 95 per cent alcohol, 1 part; ; glacial acetic acid, 5 per cent) and stained with haemotoxylin or iron haemotoxylin, after the method of histological sections. (See textbooks for methods.) Cysts of intestinal protozoa can be preserved by adding to the fecal specimen a 5 per cent aqueous solution of commercial formalin and keeping in a well-stoppered bottle.

Parasitic worms.—The intestinal worm parasites are, with few exceptions, diagnosed by their characteristic ova appearing in the feces. Direct microscopic examination of fecal specimens is made according to the directions given for the diagnosis of intestinal protozoa. *Strongyloides stercoralis* is diagnosed not by ova but by rhabditiform larvae that appear in the feces. If the fecal specimen is one or more days old, ova of the hookworm may hatch and the larvae be mistaken for those of Strongyloides.

A number of methods have been devised to concentrate the ova of intestinal worms and thus render the diagnosis quicker and more certain. They are either sedimentation or flotation methods, and of these the flotation methods appear preferable. By the Kofoid-Barber method the fecal specimen is thoroughly mixed with a concentrated brine (saturated aqueous solution of sodium chloride) in an open-mouth vessel. The coarse float is forced below the surface with a disc of No. 0 steel wool, and the specimen allowed to stand for an hour to permit the ova to ascend. The surface film is then looped off onto a slide and examined without a cover glass with low magnification. In the Willis method the sample is also mixed with concentrated brine in an open-mouth container about one inch in

diameter, and the container filled to the brim with brine. A 2 × 3 inch microscopic slide is laid across the top of the container in contact with the surface of the brine. After one-half hour to one hour the slide is removed, inverted, and examined without cover glass under low magnification, for the ova which adhere to the slide. The flotation methods are subject to certain limitations. The operculate ova of the fish tapeworm and of the flukes, and the larvae of Strongyloides do not float to the surface of the brine.

The ova of the blood fluke, *Schistosoma haematobium*, are found in the sediment of the urine, and the ova of the lung fluke, *Paragonimus westermani*, are found in the sputum of infected individuals. The larvae of *Filaria bancrofti* are found in the circulating blood. Preparation of the blood should be made at night (10 P.M. to 2 A.M.). They may be examined fresh under a cover glass, or as stained thick film preparations, as described under blood protozoa.

Ova of worm parasites are preserved by a 5 per cent aqueous solution of commercial formalin added to the material and kept in a well-stoppered bottle. Permanent microscopic slide mounts of ova can be made by adding a drop of water glass (sodium silicate) solution to the preparation, mixing, and applying cover glass. Such preparations are dissolved by water, but may be ringed with cement or varnish to render them waterproof.

The best general fixing and preserving fluid from worm parasites is 70 per cent alcohol containing 5 per cent glycerin. The worms should be fixed in the hot (not boiling) solution, and can be preserved indefinitely in it. By evaporating off the alcohol, leaving the worms in pure glycerin, the smaller worms are cleared sufficiently for study of the internal structure. The preparation of stained mounts of the larger flukes and tapeworms requires special technic, which is described in the textbooks.

Insects and arachnids are in general best preserved in alcohol. Adult flies and mosquitoes should be killed in a 'cyanide bottle' or by chloroform or tobacco smoke, and preserved between layers of absorbent cotton in small boxes or, better, pinned in tight, cork-bottom insect boxes with entomological pins. The larvae of flies and mosquitoes should be preserved in alcohol. They can then be dehydrated with absolute alcohol, cleared with xylol, and mounted in xylol-balsam.

III. The Parasitic Amoebae

Definition.—Primitive protozoa, consisting of a naked mass of nucleated protoplasm; locomotion by protrusion of pseudopodia, reproduction by simple division. An encysted stage is developed for protection during transmission from host to host. They live parasitically in the alimentary tract of man and other animals, and one species causes a serious disease of man, entamoebic dysentery, with an occasional sequela of liver abscess.

Classification.—

Subkingdom I:	Protozoa
Phylum I:	Rhizopoda
Class I:	Amoebaea
Genera:	Entamoeba
	Endolimax
	Iodamoeba
	(Councilmania)

Historical.—Read Dobell, chap. 3.*

Morphology.—Study free-living amoebae from cultures, for general morphology, motility, and life-history of amoeboid organisms. Note motile and encysted stages. Make drawings and notes.

Study fresh and stained preparations of motile and encysted stages of *Entamoeba histolytica.* Compare morphology with that of free-living amoebae. Make drawings and notes.

Compare same stages of *Entamoeba coli.* Make drawings and notes.

Read Dobell, pages 20–92; Walker (1911); Walker and Sellards (1913). Compile from your laboratory study and reading a comparative table of the differential characters of the two species.

Compare the characters of the two species studied with specimens and descriptions of other recently described species of amoebae in the intestinal tract of man: *Endolimax nana, Iodamoeba bütschlii,* and *Councilmania lafleuri.* Read Dobell, chaps. 6, 7; Kofoid and Swezy (1921); Wenyon (1922). Note the number of so-called species of amoebae parasitic in the intestinal tract of man that were proved by Walker (1911) to be identical with either *E. histolytica* or *E. coli.* There is need of further investigation of these recently described species. Compare the characters of *E. histolytica* and *E. coli* from

* References are listed in full at end of section.

the intestinal tract with those of *E. gingivalis* from the tartar of the teeth of man. Examine fresh scrapings from the junction of the teeth with the gums, especially of decayed teeth, for *E. gingivalis*.
Read: Dobell, section on *E. gingivalis;* Kofoid and Swezy (1924).

Life-history.—Multiplication by simple division of the motile stage. An encysted stage is developed for protection during transmission from man to man. No alternative host. Read Dobell, sections on life-history.

Mode of infection.—Intestinal species by ingestion of cysts with contaminated food or water; *E. gingivalis* probably by direct contagion.

Geographical distribution.—World-wide, but especially in the tropics and subtropics.

Pathogenesis.—Entamoeba histolytica is the only definitely proved pathogenic species of amoebae parasitic in man.

Study sections of large intestine from case of entamoebic dysentery and liver abscess. Note character of lesions and presence of entamoebae in the tissues. Make drawings and notes.

Read sections on symptomatology and pathology of entamoebic dysentery and entamoebic liver abscess in textbooks; Walker and Sellards (1913). Compile a brief statement of the essential symptoms and pathological changes in entamoebic dysentery and liver abscess. Note especially the prevalence of chronic infections and of convalescent and contact carriers and their significance in the dissemination of the infection.

Animal infection.—Kittens, monkeys, and rats can be infected by feeding on rectal injection. In kittens this infection causes an acute and fatal dysentery, occasionally accompanied by liver abscess. Obtain fresh feces from a human case of entamoebic infection, inoculate kittens, and study the course of the infection, clinically, microscopically, and (post-mortem) pathologically.

Diagnosis.—Read sections on diagnosis in textbooks; Walker and Sellards (1913); Dobell (1921).

Compile from your laboratory studies and reading the essential points in:

I. Laboratory diagnosis of { Intestinal entamobiasis / Liver abscess }

1. Collection of material.
2. Technic of microscopic examination.
3. Recognition of entaboeabe.
4. Differentiation of *Entamoeba histolytica* from *E. coli* and other non-pathogenic species.
5. Technic of making stained permanent preparations.

II. Clinical (symptomatic) diagnosis { Intestinal entamoebiasis / Liver abscess

Which gives the more unequivocal diagnosis, the laboratory finding or the clinical symptoms?

Prophylaxis.—Compile from your notes and reading:

1. The principal methods of dissemination of entamoebic infection.
2. The rôle of 'carriers.'
3. The most effective methods of controlling the spread of entamoebic infection.

References:

Losch, F. Massenhafte Entwickelung von Amoeben in Dickdarm. *Arch. f. path. Anat.*, 1875, 65, p. 196. (Discovery of parasitic amoebae in dysentery of man.)

Councilman, W. T., and Lafleur, H. A. Amoebic dysentery. *Johns Hopkins Hosp. Rept.*, 1891, 2, p. 393. (The etiologic relation of amoebae to one type of dysentery.)

Schaudinn, F. Untersuchungen über die Fortpflanzung einiger Rhizopoden. *Arb. kaiserl. Gesundh.-Amte,* 1903, 19, p. 547. (First distinction between a pathogenic and a non-pathogenic species in the intestinal tract of man.)

Walker, E. L. A comparative study of the amoebae in the Manila water supply, in the intestinal tract of healthy persons and in amoebic dysentery. *Philippine Jour. of Trop. Med.*, 1911, 6, p. 259. (Definite determination of life-history of pathogenic and non-pathogenic species.)

Walker, E. L., and Sellards, A. W. Experimental entamoebic dysentery. *Philippine Jour. Trop. Med.*, 1913, 8, p. 253. (Experimental determination of the parasitism and pathogenesis of *E. histolytica,* of the parasitism but non-pathogenesis of *E. coli,* and of the non-parasitism and non-pathogenesis of free-living amoebae.)

Wenyon, C. M., and O'Connor, F. W. An inquiry into some problems affecting the spread and incidence of intestinal protozoal infections, etc. *Jour. Roy. Army Med. Corps,* 1917, 28, pp. 1–6. (Description of *Endolimax nana* as a new species of intestinal amoeba of man.)

Dobell, C. *The amoebae living in man. A zoological monograph* (New York, William Wood & Co.), 1919, 6, 155 pp.. The first recognition of *Indamoeba bütschlii* as a distinct species of amoeba parasitic in the intestinal tract of man. The most complete work on the parasitic amoeba of man. Bibliography complete to date of publication.)

Kofoid, C. A., and Swezy, O. On the free, encysted and budding stages of *Councilmania lafleuri,* a parasitic amoeba of the human intestine. *Univ. Calif. Publ. Zool.*, 1921, 20, pp. 169–198.

Kofoid, C. A., and Swezy, O. The cytology of Endamoeba gingivalis etc. *Univ. Calif. Publ. Zool.*, 1924, 26, pp. 165–198.

Tropical Diseases Bulletin (London), 1912–. Review of all current literature on amoebiasis.

IV. The Leishmaniae

Definition.—The leishmaniae are round or oval, 'binucleate' protozoa, living in the endothelial cells of the subcutaneous tissues or of the liver, spleen, and bone marrow of man and the dog. In cultures and in the intestinal tract of certain blood-sucking insects the parasites become elongated and develop a single flagellum. They are probably primarily flagellate parasites of the intestinal tract of some blood-sucking insect. The Leishmaniae cause at least two distinct types of disease in man, depending upon their location. When localized in the endothelial cells of the cutaneous or mucosal tissues, they cause cutaneous or mucosal ulcerations (Oriental sore, South American cutaneo-mucosal leishmaniasis); when localized in the endothelial cells of the internal organs they cause a disease of high mortality known as kala-azar. Collectively the infections and the resulting diseases are known as leishmaniases.

Classification—

Subkingdom I:	Protozoa
Phylum II:	Mastigophora
Class I:	Flagellata
Genus:	Leishmania
Species:	tropica
	tropica var. americana
	donovani
	infantum

Historical.—Read section in textbooks on tropical medicine; Laveran (1917).

Morphology.—Study slides from cases of cutaneous and of visceral leishmaniasis. Make drawings and notes of infected cells, contained parasites, and of single free parasites. Can you distinguish any constant difference between the parasites of cutaneous and visceral leishmaniasis?

Read section on morphology in textbooks and compare plates with your drawings. Read recent literature (reviewed in *Trop. Dis. Bull.*) on the possible identity of the parasites of the several types of leishmaniasis. There is need of further investigation on this subject.

Life-history.—The rounded parasites in the endothelial cells multiply by simple division until the distended cell ruptures, setting free the parasites which penetrate other endothelial cells. In visceral

leishmaniasis the parasitized endothelial cells and free parasites are found in small numbers in the circulating blood. In cultures on special blood-agar medium the rounded parasites become elongated, develop a single flagellum at the anterior end, and become actively motile. It is presumed that this motile stage represents the stage of life-history occurring in the gut of some blood-sucking insect that serves as an alternative host of the parasite; but definite proof of this is wanting.

Study stained preparations and living organisms from cultures of *Leishmania infantum* and *L. tropica.* Compare with the Herpetomonad parasite from the gut of the dog flea. Make drawings and notes. Can you distinguish any differences between the cultural forms of *L. infantum,* and *L. tropica* and *H. etenocephali?*

Read sections in textbooks and special articles on the life-history of the leishmaniae.

Cultivation.—The leishmaniae are cultivable on a special blood-agar medium, Novy-McNeal-Nicolle medium. Make cultures of *L. infantum* and *L. tropica* and follow their development.

Mode of Infection.—Read references on the transmission of the leishmaniases with special attention to:

1. Basil's theory of the flea transmission of Mediterranean kala-azar.
2. Donovan's theory of the bedbug transmission of Indian kala-azar.
3. Sergeant's theory of the sand-fly transmission and lizard reservoir of Oriental sore.
4. Fanthan's theory of the origin of the leishmaniases from strayed intestinal flagellates of blood-sucking insects.

The need for further investigation of the problems of the life-history and transmission of the leishmaniases is evident.

Pathogenesis. Types of disease.

1. Cutaneous—Oriental sore—*L. tropica.*
2. Cutaneo-mucosal—South American leishmaniases—*L. tropica* var. *Americana.*
3. Visceral: { Mediterranean kala-azar—*L. infantum*
   { Indian kala-azar—*L. donovani*

Read references on pathogenesis of the leishmaniae with special attention to:

1. Clinical manifestations of the several types of disease.
2. Pathological changes of the several types of disease.
3. Mortality of the several types of disease.

4. Evidence for and against the plurality of species of the parasite of the several types of disease.

Spontaneous visceral leishmaniasis occurs in dogs in the Mediterranean region. The leishmaniae are inoculable into the dog, mouse, and rat, but the percentage of failure to infect is large.

Geographical Distribution—

1. Oriental sore—tropical Asia, Africa, and Mediterranean Europe, but distribution is not uniform.
2. Cutaneo-mucosal—tropical America.
3. Mediterranean kala-azar—Mediterranean basin.
4. Indian kala-azar—India, China, Asia Minor.

Diagnosis.—

I. Laboratory.

A. Cutaneous and cutaneo-mucosal leishmaniasis.

Technic of obtaining material, making and staining preparations, and examination of preparations for Leishmania parasites.

B. Visceral leishmaniasis.

1. Technic of obtaining material for microscopic examination from:
   (*a*) Spleen puncture.
   (*b*) Liver puncture.
   (*c*) Bone marrow puncture.
   (*d*) Cervical gland puncture.
   (*e*) Blood.
2. Technic of blood cultures.
3. Technic of the formol-gel test.

There is need of further investigation of the formol-gel test for kala-azar. Draw conclusions as to the most efficient method of laboratory diagnosis of visceral leishmaniasis, and the relative danger of the several procedures to the patient.

II. Clinical.

Note the essential clinical symptoms of cutaneous, cutaneo-mucosal, and visceral leishmaniasis from your reading, and compare the reliability of the laboratory and the clinical methods of diagnosis.

Prophylaxis.—The leishmaniases present a striking example of the results of an incomplete knowledge of the life-history of a parasite upon rational prophylaxis. From your notes on the known facts of the life-history of the leishmaniae, deduce provisional prophylactic measures.

References:

LEISHMAN, W. B. On the possibility of the occurrence of trypanosomiasis in India. *Brit. Med. Jour.*, 1903, May 30, pp. 1252–1254. (Discovery of the parasite of kala-azar.)

DONOVAN, C. Human piroplasmosis. *Lancet*, 1904, 2, p. 744. (Discovery of the parasite of kala-azar.)

WRIGHT, J. H. Protozoa in a case of tropical ulcer (''Delhi sore''). *Jour. Med. Res.*, 1903, 10, pp. 472–482. (Discovery of parasite in cutaneous leishmaniasis.)

ROGERS, L. Cultivation of the protozoal parasite of cachexial fever, and the development of trypanosoma from Cunningham-Leishman-Donovan bodies. *Brit. Med. Jour.*, 1904, No. 2281, pp. 648–650. (First cultivation of flagellate from the kala-azar parasite.)

CARINI, A., ET PARANPHOS, W. *Soc. de path. exotique*, 1909, May 12. (Discovery of the parasite of cutaneo-mucosal leishmaniasis of South America.)

NAPIER, L. E. A new serum test for kala-azar. *Indian Jour. Med. Res.*, 1922, 9, pp. 803–946; *Trop. Dis. Bull.*, 1922, 19, pp. 546–547.

Tropical Diseases Bulletin (London), 1912–. (Reviews all articles on leishmaniases to date.)

V. THE TRYPANOSOMAE

Definition.—Lancet-shaped protozoa, having as organs of locomotion an undulating membrane, along the border of which is attached a single flagellum that extends free at the anterior end of the parasite. They live parasitically in the blood plasma of man and other animals, and some of them cause fatal disease in their hosts. They are for the most part transmitted by blood-sucking insects.

Classification.—

Subkingdom I:	Protozoa
Phylum I:	Mastigophera
Class I:	Flagellata
Family:	Trypanosomidae
Genus:	Trypanosoma
Species (in man)	Tr. gambiense
	Tr. rhodesiense
	Tr. cruzi

Historical.—Read section in textbooks.

Morphology.—Examine fresh blood preparations from guinea pig infected with *Trypanosoma brucei.* Make film preparations of blood and stain with Wright's or Giemsa's stain. Repeat until technic is perfected.

Make drawings and notes on the morphology of the parasites from your stained preparations.

Study stained preparations of *Tr. gambiense*, *Tr. cruzi*, and trypanosomes of lower animals, and compare with those of *Tr. brucei* prepared by yourself.

Read sections on morphology in textbooks and note differential characters among the three species infecting man.

Life-history.—The trypanosomes, with few exceptions, have two hosts, one a vertebrate and the other a blood-sucking invertebrate. In the vertebrate host the trypanosomes multiply in the blood by longitudinal division, and may develop rounded aflegellate forms similar to the leishmaniae in the internal organs. When ingested with the blood by the proper, and usually specific, invertebrate host, the trypanosomes multiply and undergo a certain development in the gut of the alternative host. The parasite is inoculated into a fresh vertebrate host by the infected invertebrate, either in the process of biting and blood-sucking, or, in some species, by the contamination of the bite wound with feces of the invertebrate. The outstanding exception to this life-history is that of *Trypanosoma equiperdum*, causing a disease of horses known as dourine, which has no alternative host but is transmitted from horse to horse by sexual intercourse, like syphilis in man.

The trypanosomes can be cultivated with difficulty on special blood-agar media.

Dissect the louse-fly of the sheep and examine intestinal contents for flagellates. Make stained smears. Study the morphology of the forms found. Make notes and drawing. This parasite is a stage of the trypanosome living in the blood of the sheep, of which the louse-fly is the alternative host.

Read section in textbook and special articles on the life-history of trypanosomes, with special references to species parasitic in man.

Mode of infection.—Tr. gambiense is transmitted by the bites of infected tsetse flies of the species *Glossina palpalis.*

Tr. rhodesensis is transmitted by the bites of infected tsetse flies of the species *Glossina morsitans.*

Tr. cruzi is transmitted by the bites of infected blood-sucking bugs of the species *Conorhinus megistus.*

Examine specimens and illustrations of *Glossina* and *Conorhinus.* Read section on transmission of trypanosomes in textbooks and special articles.

Geographical Distribution.—

Tr. gambiense—Central Africa.

Tr. rhodesiense—Rhodesia, South Central Africa.

Tr. cruzi—Brazil, South America.

Pathogenesis.—

Tr. gambiense and *Tr. rhodesiense* cause African sleeping sickness in man.

Tr. cruzi causes a peculiar type of trypanosomasis in man in South America. A number of species of trypanosomes cause diseases of animals. The human species are inoculable into animals.

Read sections in textbooks on symptomatology and pathology of African and of South American trypanosomiasis.

Diagnosis.—

I. Laboratory.

A. By microscopic examination of the blood.

(1) Direct.

(2) After centrifuging blood.

B. By inoculation of susceptible animals with blood of suspected patient.

C. By microscopic examination of material from cervical gland puncture.

D. Relative value of the four methods.

Inoculate a guinea pig with *Tr. brucei* and follow appearance of trypanosomes in the circulating blood and their presence or absence from day to day.

II. Clinical. Read sections in textbooks and construct a table of the essential symptoms. Consider the relative reliability of the laboratory and symptomatic diagnosis of trypanosomiasis.

Prophylaxis.—It will be recalled that in amoebic dysentery, there being no alternative host or definitely known reservoir of the infection, prophylaxis is restricted to the control of the human carrier. In the leishmaniases, an alternative host although probable is not definitely known and no reservoir has been established, therefore, again, prophylaxis is largely restricted to the human carrier. In contrast to the foregoing infections, the trypanosomiases of man have definitely known alternative hosts and animal reservoirs. Therefore they present three distinct points of prophylactic attack: the human carrier, the insect transmitter, and the animal reservoir. Prophylaxis may consequently be directed against:

1. The human carrier, by segregation of the sick, by protecting them from bites of the transmitting insect, or by treatment to render them non-infective.

2. The insect vector, by destruction of the insect and its breeding places.

3. The reservoir, by extermination of wild animals that are capable of becoming infected.

Read section on prophylaxis in the textbooks and special articles.

References:

Dutton, J. E. Preliminary note upon a trypanosome occurring in the blood of man. *The Thomson Yates Lab. Rept.,* 1902, 4, p. 455. (First observation of *Tr. gambiense* in the fever stage of sleeping sickness, not then recognized as an early stage of sleeping sickness.)

Castellani, A. On the discovery of a species of trypanosoma in the cerebrospinal fluid of a case of sleeping sickness. *Lancet,* 1903, p. 1735.

Chagas, C. Nova trypanozomiaze humana (Uber eine neue Trypanosomiases des Menschen). *Memoras do Instituto Oswaldo Cruz,* 1909, 1, pp. 160–218. (Discovery of the parasite of human tryponosomiasis of Brazil and its transmission by *Conorhinus megistis.*)

Stephens, J. W. W., and Fantham, H. B. On the peculiar morphology of a trypanosome from a case of sleeping sickness and the possibility of its being a new species (*Tr. rhodesiense*). *Proc. Roy. Soc.,* 1910, Ser. B, 83, No. 561, pp. 28–33.

Bruce, D., Nabarro, D., and Griegg, E. D. W. *Reports of the Sleeping Sickness Commission of the Royal Society,* No. 4, 1903 (London, Harrison and Sons). (Showed that *Tr. gambiense* was spread by *Glossina palpalis.*)

Kleine. Weitere Untersuchungen über die Atiologie der Schlafkrankheit. *Deutsche med. Wochnschr.,* 1909, p. 1257. (Showed that *Tr. gambiense* undergoes a definite cycle of development in *Glassina palpalis.*)

Kinghorn, A., and Yorke, W. On the transmission of human trypanosomiasis by *Glossina morsitans. Am. Trop. Med. and Parasitol.,* 1912, 6, A, pp. 1–23. (Showed that *Tr. rhodesiense* was transmitted by, and undergoes a definite cycle of development in, *G. morsitans.*)

Tropical Diseases Bulletin (London), contains references and comprehensive reviews of all literature on trypanosomiasis.

VI. The Intestinal Flagellates of Man

Definition.—Small pear-shaped, multiflagellate protozoa living as parasites in the intestinal tract of man.

Classification.—

Subkingdom I:	Protozoa
Phylum II:	Mastigophora
Class I:	Flagellata
Genus and species 1:	*Trichomonas hominis*
Genus and species 2:	*Chilomastix mesnili*
Genus and species 3:	*Giardia intestinalis*

Historical.—Read sections in textbooks.

Morphology.—Study fresh and stained preparations of *Trichomonas, Chilomastix,* and *Giardia* in the motile and the encysted stages. Determine the distinguishing characters of each species. Make drawings and notes.

Read: Dobell and O'Connor (1921); Kofoid and Swezy (1921). Compare descriptions and figures with your own drawings.

Life-history.—Motile stage multiplies by longitudinal division. An encysted stage is usually developed for protection during transmission from man to man. No alternative host.

Mode of infection.—By ingestion of the encysted parasites with contaminated food or water.

Geographical distribution.—World-wide, but especially prevalent in the tropics and among people of low sanitary development.

Pathogenesis.—Doubtful. Read section on pathogenesis in Dobell and O'Connor (1921), Kofoid and Swezy (1921), Fantham, Stephens and Theobald (1916), pp. 620–625, special articles.

Diagnosis.—By microscopic examination of fresh stools and identification of the motile or encysted flagellates. *Giardia intestinalis* is usually found in the characteristic encysted stage. The other two species in the motile stage.

Read section on diagnosis in Dobell and O'Connor (1921).

Prophylaxis.—Proper disposal of feces; avoidance of contaminated food and water.

References:

Dobell, C., and O'Connor, F. W. *The intestinal protozoa of man* (William Wood and Co., N. Y., 1921).

Kofoid, C. A., and Swezy, O. The flagellate infections of the human digestive tract. *Nelson's Loose Leaf Encyclopedia of Living Medicine,* 1921, 5, pp. 365–398.

Tropical Diseases Bulletin, 1912–. Review of all articles on intestinal flagellates.

VII. The Intestinal Ciliates

Definition.—Relatively large and highly organized unicellular animals. Instead of a few long whiplash-like organs of locomotion, as in the flagellates, the ciliates have a large number of fine cilia more or less covering the surface of the cell, by the movement of which, they swim about. They possess two nuclei, a macronucleus and a micronucleus, a cytostome and cytopharynx and primitive excretory organs, the contractile vacuoles. All of these organelles are not of

multicellular structure, but are differentiations of the protoplasm of the unicellular organism. They exhibit a primitive sexual process, conjugation. The parasitic forms live for the most part in the intestinal tract of man and other animals, and one of them, *Balantidium coli,* may cause a fatal disease of man, balantidial dysentery.

Classification.—

Subkingdom I:	Protozoa
Phylum III:	Ciliophora
Class I:	Ciliata
Genus and species:	*Balantidium coli*

Historical.—Read section in textbooks.

Morphology.—Study stained preparations and preserved material of *Balantidium coli.* Make drawings and notes of motile and encysted parasites.

Obtain fresh feces from pig and study living motile and encysted parasites.

Read sections in textbooks and compare figures and description with your own drawings and notes.

Life-history.—*Balantidium coli* multiplies by *transverse* division. A primitive sexual process, conjugation, takes place in many of the ciliates and has been described for *Balantidium coli.* The parasite has an encysted stage for protection during transmission from host to host.

No alternative host is necessary for its life-cycle.

The domestic pig is the reservoir of the infection in man.

Read section on life-history in textbooks with special reference to details of division and the physiological significance of conjugation.

Mode of infection.—By ingestion of food or water contaminated with cysts of *Balantidium coli.* The domestic pig is probably the normal host of this parasite and man becomes infected directly or indirectly from the pig.

Geographical distribution.—World-wide in the pig; relatively infrequent in man.

Pathogenesis.—*Balantidium coli* may cause a fatal dysentery in man, but the proportion of 'carriers' of the parasite that show no clinical symptoms, at least for long periods of time, are even greater than in the case of infections with *Entamoeba histolytica;* however, every infected person is liable eventually to develop a fatal dysentery. In its normal host, the pig, *Balantidium coli* appears to have little or no pathologic action. The parasite is inoculable into monkeys, in

which it is capable of penetrating the tissues and causing intestinal lesions. Guinea pigs can be infected by rectal injections of feces containing balantidia and the animals are convenient for the study of the parasites.

Study sections of large intestine of a fatal case of balantidial dysentery. Make drawings and notes of lesions and the parasites in the tissues.

Read Strong (1904), Bowman (1909, 1911), von Prowazek (1913), Walker (1913), Dobell and O'Connor (1921) with especial reference to pathology and symptomatology.

Diagnosis.—By microscopic examination of the stools for the parasite. In the feces of man the parasite is always found in the motile stage; in feces of the pig both motile and encysted forms occur. Infections of man are characterized by the intermittent appearance of the parasites in the stools; they may therefore be overlooked unless repeated examinations are made.

Prophylaxis.—As infection comes from the domestic pig, precautions should be taken to prevent direct or indirect contamination of food or water with fecal matter from the pig, also hand to mouth infection of those who come in intimate contact with pigs or their excrement.

References:

Malmsten. Infusorien als Intestinaltiere bei Menschen. *Arch. f. pathol. Anatomie,* 1857, 12, p. 302. (Discovery of *Balantidium coli* as a parasite of man.)

Strong, R. P. The clinical and pathological significance of *Balantidium coli.* Dept. Interior, Bureau of Govt. Lab., Biol. Lab., *Bull.* No. 26, December, 1904.

Bowman, F. B. Two cases of *Balantidium coli* infection. *Philippine Jour. Sci.,* Sec. B, 1919, 4, pp. 417–425. A case of dysentery caused by Balantidium coli with coincident filarial infraction of the spleen. *Ibid.,* 1911, 6, pp. 147–153.

Von Prowazek, S. Zur Kenntnis der Balantidiosis. *Archiv. f. Schiffs- und Tropenhygiene,* 1913, 17, Beiheft 6, pp. 371–390.

Walker, E. L. Experimental balantidiasis. *Philippine Jour. Trop. Med.,* 1913, 8, 333–347.

Dobell, C., and O'Connor. *The Intestinal Protozoa of Man.* Wm. Wood and Co., New York, 1921.

Tropical Diseases Bulletin (London), 1912–. Review of all articles on balantidiasis to date.

VIII. The Coccidia

Definition.—Protozoa living as parasites within the epithelial cells of higher animals. Living intracellularly they have no need for organs of locomotion. The coccidia have, however, a relatively complex life-history with an alternation of an asexual and a sexual reproduction. They inhabit the epithelial cells of the intestinal tract and bile ducts of the liver, for the most part, but certain species live in the epithelial cells of the urinary tubules in the kidney. They are relatively infrequent parasites of man and appear not to be of pathological significance.

Classification.—

Subkingdom I:	Protozoa
Phylum IV:	Sporozoa
Class I:	Telosporidia
Order II:	Coccidia
Genus I:	Isospora
Genus II:	Eimeria

Historical.—Read section in textbooks.

Morphology and life-history.—Study stained sections of intestine and preserved fecal material containing *Isospora* and *Eimeria* and identify stages by diagram of life-cycle of coccidia in textbooks. Make drawings and notes.

Obtain feces from rabbit infected with coccidia and culture. Study development of sporozoites in the oöcysts.

Read sections on morphology and life-history of the coccidia in textbooks.

Mode of infection.—By ingestion of ripe oöcysts containing mature sporozoites with contaminated food or water.

Geographical distribution.—Infections of man are few and scattered. Read sections on reported human cases in textbooks.

Pathogenesis.—Since the coccidia live within epithelial cells of the small intestine, which they destroy in their development, and are constantly multiplying and infecting new epithelial cells, they must produce some pathological effects. In man, however, these pathological effects do not appear to be sufficient to cause recognizable clinical symptoms. In some other animals coccidiasis produce well-recognized disease.

Read section on pathogenesis in Dobel and O'Connor (1921).

Diagnosis.—By microscopic examination of the feces and discovery of the oöcytes.

Prophylaxis.—Since it is not known whether infection is derived from other human cases or from animal reservoirs, it is not possible to recommend specific prophylactic measures.

Reference:

DOBELL, C., AND O'CONNOR, F. W. *The Intestinal Protozoa of Man.* Wm. Wood and Co., New York. (Contains all that is known of the species parasitic in man with full bibliography.)

IX. THE MALARIAL PARASITES

Definition.—Protozoa living parasitically in the red blood corpuscles of vertebrates and causing the several types of malarial fever in man. They are transmitted by the bites of infected mosquitoes which are the alternative hosts of these parasites.

Classification.—

Subkingdom I:	Protozoa
Phylum IV:	Sporozoa
Class I:	Telesporidia
Order III:	Haemosporidia
Genus I	Plasmodium
Species 1:	vivax
Species 2:	malariae
Species 3:	falciparum

Historical.—Read section on history in textbooks with special reference to:

1. The early miasma theory of the etiology of malaria.
2. The succeeding bacterial theory.
3. Laveran's (1880) discovery of the malarial parasite.
4. The theories of Manson, Bigami, and Koch concerning the method of transmission of the infection.
5. Ross (1897–98). The life-history of the malarial parasite of birds in the culecine mosquito and its transmission from bird to bird by bites of infected mosquitoes.
6. Grassi (1901). The demonstration of a similar life-history for the human malarial parasites in the anopheline mosquito.

7. Low, Sambon and Manson (1900). The experimental proof of the mosquito transmission of malaria to man.
8. Golgi (1886–89). The discovery of the plurality of species of malarial parasites.

Morphology.—Study stained blood smears containing the three species of malarial parasites, *P. vivax*, *P. malariae*, and *P. falciparum*, with special reference to the trophozoite, schizogony, gametes, and the differential characters of the three species. Make drawings and notes. Endeavor to find a case of malaria in some of the hospitals, from which obtain fresh blood preparations and study the living malarial parasites. Make dry film preparations and stain them.

Read in the textbooks the description of the stages of the three species of malarial parasites occurring in the blood of man. Compare descriptions and figures with your own observations and drawings.

Life-history.—The malarial parasites have a complex life-history with an alternation of generations and of hosts. In man, who is the intermediary host, the parasite multiplies asexually by multiple division (schizogony), producing merozoites which infect other blood corpuscles. The parasite also develops in man the gametes preparatory to the sexual reproduction in the mosquito. In the anopheline mosquito, which is the definitive host, the gametes, ingested with the blood from infected man, mature and copulate. The resulting zygote, becoming encysted in the wall of the gut of the mosquito, multiplies by sporogony and produces falciform sporozoites which, set free by the rupture of the ripe oöcysts into the body cavity, enter the salivary glands. These sporozoites in the salivary glands of the mosquito are injected into the circulating blood when an infected mosquito bites man.

Read description of the life-history of the malarial parasite in the textbooks and study diagram of life-cycle.

Mode of infection.—Man is infected by the bites of infected anopheline mosquitoes, the mosquito is infected by sucking the blood of infected man; neither man nor mosquito can become infected in nature in any other way. Man can be infected experimentally by injections of blood from another infected man. Only the anopheline mosquitoes can be infected by and transmit the parasites of human malaria; the culecine mosquitoes are immune.

Read: Schuffner (1902), James (1902), Darling (1910), Stanton (1912), Knab (1913), Walker and Barber (1913).

Study specimens of adult and larvae of anopheline and culecine mosquitoes with special reference to differential characters. Learn to recognize anopheline mosquitoes both in the adult and the larval

stage. The importance of this knowledge will be apparent when we come to consider the prophylaxis of malaria.

Collect mosquito larvae and study development of mosquito in the laboratory.

Read in textbooks the descriptions of the anopheline and culecine mosquitoes and their life-histories.

Geographical distribution.—Malaria occurs wherever conditions are suitable for the continued propagation of anopheline mosquitoes. This includes tropics and subtropics, with extensions into the temperate zone.

Pathogenesis.—There are 3 types of malarial fever in man, corresponding to the 3 species of malarial parasites and presenting differential clinical manifestations.

Read sections on symptomatology and pathology of malaria in textbooks with special reference to the differential characters of the types of disease, acute and chronic malaria, malarial relapses, and the relation of these clinical manifestations to the life-history of the parasites.

Endeavor to locate a case of malarial fever in the hospitals and follow course of the disease.

The 3 species of malarial parasites of man cannot be inoculated into lower animals; but many of the lower animals suffer naturally from infections with other species of malarial parasites.

Diagnosis.—

I. Laboratory: Work out from the laboratory studies and reading a procedure for making a laboratory diagnosis of malaria, including:

1. Collection of blood:
   Technic.
   Time.
2. Making preparations:
   Thick film.
   Thick smear.
3. Fixing and staining preparations.
4. Examination of preparations.
5. Interpretation of observations:
   Malarial parasites.
   Species of parasite.
   Other clinical manifestations in the blood.

II. Clinical. Compile from reading and observation the essential points in the symptomatology of malaria and the differential symptoms of the 3 types of the disease.

III. Therapeutic. Fevers that yield to quinine treatment are malarial in etiology.

Prophylaxis.—Malaria being transmitted by a specific alternative host, the anopheline mosquito, its control offers 3 points of attack at which we may interrupt the life-cycle of the parasite, namely: (1) the malarial patient, (2) the anopheline mosquito, and (3) the uninfected population.

Theoretically the breaking of any one of these 3 links in the chain of the life-cycle of the malarial parasite, if complete and world-wide, would exterminate malaria from the world. In practice it is usually necessary to attack all 3 vulnerable points in the life-cycle of the parasite, and then, unless persistent, the conquest fails, even in restricted localities.

Read section on prophylaxis in textbooks; Boyce (1909), Bass (1919), *Trop. Dis. Bull.*, Sanitation Supplements, of recent years.

Compile a scheme for prophylaxis of malaria with special reference to following details:

1. Malarial survey of region:
   - *a.* Endemic index { Morbidity rate; Spleen rate; Parasite rate
   - *b.* Mosquito index { Breeding places; Species breeding
2. Methods of attack from the point of view of:
   - *a.* Infected persons.
   - *b.* Anopheline mosquitoes.
   - *c.* Uninfected population.

References:

LAVERAN, A. Un nouveau parasite trouvé dans le sang des malades atteints de fievre palustre etc. *Bull. et Mem. de la Soc. Med. des Hopitaux de Paris,* 1880, 17.

ROSS, R. On some peculiar pigmented cells found in two mosquitoes fed on malarial blood. *Brit. Med. Jour.,* 1897, 2, pp. 1786–1788.

ROSS, R. The rôle of the mosquito in the evolution of the malarial parasite. *The Lancet,* London, 1898, 2, pp. 488–489.

GRASSI, B. Preliminary communications in *Rend. R. Accad. d. Lincei,* Roma, ser. 5, 1898, 7, pp. 163, 234, 314; 1899, 8, p. 165. (Announcement of the discovery of a development of the human malarial parasite in anopheline mosquitoes similar to that of the parasite of bird malaria in culecine mosquitoes as described by Ross.)

MANSON, P. Experimental proof of the mosquito-malaria theory. *The Lancet*, London, 1900, p. 923.

DARLING, S. T. Factors in the transmission and prevention of malaria in the Panama Canal Zone. *Ann. Trop. Med. and Parasitol.*, 1910, 4, pp.179–223.

BOYCE, R. *Mosquito or man?* E. P. Dutton and Co., New York, 1909.

KNAB, F. The species of anopheles that transmit human malaria. *Jour. Trop. Dis. and Prevent. Med.*, 1913, 1, pp. 33–43.

WALKER, E. L., AND BARBER, M. A. Malaria in the Philippine Islands, I. Experiments on the transmission of malaria with *Anopheles* (*Myzomzia*) *febrifer* sp. nov., etc. *Philippine Jour. Sci.*, Sec. B, *Trop. Med.*, 1914, 9, pp. 381–439.

BASS, C. C. Studies on malaria control. *Southern Med. Jour.*, 1919, 12, p. 456; *Jour. Am. Med. Assoc.*, 1919, 72, p. 1218; *Southern Med. Jour.*, 1919, 12, p. 190; *Osler Memorial Volume*, Paul B. Hoeber, N. Y.; *Southern Med. Jour.*, 1919, 12, pp. 306, 460, 462, 465; *Jour. Am. Med. Assoc.*, 73, p. 21; *Jour. Am. Public Health Assoc.*, 1920; *Southern Med. Jour.*, 1921, 13, p. 250. (A series of 11 papers on malaria control work conducted jointly by the International Health Board and the Mississippi State Board of Health.)

LANE, C. Malaria: A critical review. *Trop. Dis. Bull.*, 1922, 19, 93–102; The malarias. Recent studies and their bearings. *Trop. Dis. Bull.*, 1923, 20, pp. 771–779.

Reviews of all papers published in any language on malaria will be found in the current numbers of the *Tropical Diseases Bulletin*, London.

X. THE TREMATODA

Definition.—Flat, leaf or tongue-shaped, bilaterally symmetrical, unsegmented worms, living parasitically in the small intestine, bile ducts of the liver, lungs, or portal circulation of man and other animals. Some of them cause serious diseases.

Classification.—

Subkingdom II:	Metazoa (multicellular animals)
Phylum VII:	Plathelminthes (flat worms)
Class II:	Trematoda (flukes)

Numerous families, genera, and species.

Historical.—See Fantham, Stephens and Theobald (1916), introductory chapter and references under species of Trematoda.

Morphology.—Study mounted and stained preparations and preserved specimens of flukes with special reference to shape, size, suckers, digestive, reproductive and excretory organs. Make notes and sketches.

Read section on morphology of flukes in Fantham, Stephens and Theobald (1916).

Life-history.—The flukes parasitic in man have very complex life-histories. There is an alternation of one or several asexual generations in one or more intermediary hosts, with a sexual generation in man who is the definitive host.

In connection with the life-history of the flukes, the first group of worm parasites, it is convenient to call attention to a fundamental difference between the life-history of the worm parasites and of the protozoan parasites in man and other vertebrate hosts, which has a profound influence on the pathogenesis of these two groups of parasites. The protozoan parasites tend to multiply indefinitely, like the bacteria, in the infected individual, and consequently produce a progressive and often fatal disease. On the other hand, the worm parasites, with one exception, reproduce sexually one generation only, the products of which, usually eggs, are excreted and serve to infect other hosts. Consequently there is no multiplication of the worm parasites in the patient, and the severity of the pathological changes produced depends upon the number of ova or larvae originally taken into the body, or upon repeated reinfections.

Read section on development of trematodes in Fantham, Stephens and Theobald with special reference to type life-history, and variations from it. The life-history of the more important species infecting man will be considered more in detail under these species.

Obtain ova from case of fluke infection and cultivate miracidia.

Collect fresh-water snails and dissect for stages of asexual generations.

The More Important Trematode Parasites of Man

A. INTESTINAL FLUKES

1. *Fasciolopsis buskii*—

Morphology.—Study specimens of adult fluke with reference to shape, size, suckers, and structure of internal organs. Make drawings and notes. Read sections on *Fasciolopsis buskii* in textbooks.

Life-history.—Intermediary host, certain fresh-water snails; cercariae become encysted on grass; man and pig the definitive host. Read Nakagawa, K. (1921).

Mode of infection.—Man becomes infected by ingesting the encysted cercariae.

Geographical distribution.—China especially; there are said to be a million cases in one province.

Pathogenesis.—May cause fatal illness. Read Cole, A. F. (1921); Goddard, F. W. (1919).

Diagnosis.—By finding characteristic ova in stools. Study ova with special reference to shape, size, cap, and contents.

2. *Metagonimus yokogawai*—

Morphology.—See Fantham, Stephens and Theobald.

Life-history.—First intermediary host, a fresh-water snail; second intermediary host, fresh-water fish; definitive hosts, man and other mammals. Read Muto, M. (1918).

Mode of infection.—Man becomes infected by ingesting the cercariae encysted, in the muscles of infected fish.

Geographical distribution.—Especially in Japan.

Pathogenesis.—Causes chronic intestinal catarrh and local destruction of intestinal glands.

Diagnosis.—Ova in feces. Study ova of *Metagonimus yokogawai* and compare with ova of *Fasciolepsis buskii.*

B. LIVER FLUKES

3. *Clonorchis sinensis*—

Morphology.—Study preserved specimens and stained preparations of this fluke and compare with other species studied. Read section on *Clonorchis* in Fantham, Stephens and Theobald.

Life-history.—First intermediary host, a fresh-water snail, *Bythinia striatula* var. *japonicum* (in Japan), second intermediary host, certain fresh-water fish; definitive hosts, man, dog, cat. Read Kobayshi, H. (1917).

Mode of infection.—Man becomes infected by ingestion of cercariae encysted in the muscles of infected fish.

Geographical distribution.—China and Japan especially. The Chinese and the Japanese species have been claimed to be distinct.

Pathogenesis.—Produces a severe chronic disease of the liver. Read Mebius, J. (1920).

Diagnosis.—Ova in the feces. Study ova in preserved feces and compare with ova of other species studied, especially with ova of *Metagonimus yokogawai.*

4. *Opisthorchis felineus*—

Morphology.—Study stained specimen and compare with other species of flukes studied. Read description in Fantham, Stephens and Theobald.

Life-history.—A probable first intermediary host is as yet unknown; second intermediary host, fresh-water fish; definitive hosts, man, cat, dog, fox. Read section on life-history in Fantham, Stephens and Theobald.

Mode of infection.—By ingestion of cercariae encysted in the muscles of infected fish.

Geographical distribution.—Siberia and western Europe.

Pathogenesis.—Causes more or less extensive pathological changes in the liver.

Diagnosis.—Ova in feces. Study ova of *Opisthorchis* in preserved feces and compare with ova of other species studied.

C. LUNG FLUKES

5. *Paragonimus westermani.*

Morphology.—See Fantham, Stephens and Theobald.

Life-history.—First intermediary host a fresh-water snail of the genus *Melania;* second intermediary hosts a land crab or a crayfish; definitive hosts man, dog, cat, etc. Read Nakagawa, K. (1916); Kobayashi, S. (1921).

Mode of infection.—By ingestion of cercariae encysted in the muscles of infected crabs or crayfish.

Geographical distribution.—China, Korea, and especially Japan.

Pathogenesis.—This fluke localizes in the lungs, pleura, and especially in the bronchi. There are claims that the parasite may also have a wide distribution in the other organs and tissues of the body. The worms and their ova cause catarrhal, hemorrhagic, and purulent bronchitis and peribronchitis, and areas of consolidation and cirrhosis of the lung. Infections may be mistaken for pulmonary tuberculosis. Read Fantham, Stephens and Theobald (1916); Musgrave, W. E. (1907); Kawamura, R., and Yaamaguchi, M. (1921); Kimura, O. (1921).

Diagnosis.—Ova in the sputum. Study ova of Paragonimus in preserved sputum. Note characteristics and make drawing.

D. BLOOD FLUKES

6. *Schistosoma japonicum*—

Morphology.—Study mounted specimens of the parasite. Note the separate sexes (found only in the Schistosomidae of Trematoda) and other differences from the flukes hitherto studied. Read section in Fantham, Stephens and Theobald (1916).

Life-history.—Alternative host, fresh-water snails (in Japan, *Katayama nosophora*); cercaria, escaping from the snail, swim free in water; definitive hosts, man, dog, cat, cattle, etc. Read Leiper, R. T., and Atkinson, E. L. (1915).

Mode of infection.—Chiefly by the penetration of the free-swimming cercariae through the skin of persons bathing or wading in infected water; infection by the way of the intestinal tract from drinking infected water is also a possibility.

Geographical distribution.—Japan; China, especially in the Yangtze Valley; Formosa.

Pathogenesis.—The pathogenesis of *Schistosoma japonicum* is due to the penetration of the larvae through the skin, the wandering of the larvae through the body, the presence of the adult worms in the portal circulation, and especially to the ova, which cause inflammatory reactions and ulcerations. Read Lanning (1914); Fantham, Stephens and Theobald (1916); Man (1916). Study sections of liver and intestine of a case of schistosomiasis, noting especially the ova, their position in the tissues and cellular reactions.

Diagnosis.—The ova of *Schistosoma japonicum* escape through ulcerations into the intestinal tract and are excreted with the feces. Diagnosis by discovery of the ova in the stools. Examine specimen of preserved feces for ova and study their characteristics. Make notes and drawings. Read section on diagnosis in textbook.

7. *Schistosoma haematobium*—

Morphology.—Consult Fantham, Stephens and Theobald (1916). Compare with *S. japonicum.*

Life-history.—Similar to *S. japonicum.* Read Leiper, R. T. (1915).

Mode of infection.—The same as in case of *S. japonicum.*

Geographical distribution.—Africa especially, Arabia, Greece, Cyprus.

Pathogenesis.—The spined ova produce chronic inflammatory lesions of the bladder and urogenital organs. Read sections on pathology and symptomatology in textbooks.

Diagnosis.—Ova escape by the way of the urinary tract and are to be looked for in the urine, which often contains pus. Compare with *S. japonicum.* Study mounted slide of ova of *S. haematobium* and compare with ova of *S. japonicum.* Make notes and drawings.

8. *Schistosoma mansoni*—

Morphology.—Read textbooks.

Life-history.—Similar to *S. japonicum* and *S. haematobium.* Read Lutz (1916); Gonzales, E. (1917).

Mode of infection.—Similar to *S. japonicum* and *S. haematobium.*

Geographical distribution.—Tropical America, Africa.

Pathogenesis.—Similar to *S. japonicum.*

Diagnosis.—Ova escapes by way of feces as is the case with *S. japonicum.*

Contrast with *S. haematobium.*

Examine preserved feces for ova of *S. mansoni.* Compare with ova of *S. japonicum* and *S. haematobium.* Make notes and drawings.

Prophylaxis of trematode infections.—In the case of the schistosomiases, which have swimming cercariae, avoid bathing, wading, or drinking of infected water in endemic regions. In case of the trematodes, in which the cercariae encyst in the muscles of fish or shellfish, avoid eating raw or insufficiently cooked fish or shellfish in endemic regions.

References:

Nakagawa, K. On the life-cycle of *Fasciolopsis buski* Lankester. *Kitasato Arch. Exper. Med.,* 1921, 4, pp. 159–167; *Trop. Dis. Bull.,* 1922, 19, p. 215.

Goddard, F. W. *Fasciolopsis buski,* a parasite of man as seen in Shaohing, China. *Jour. Parasitol.,* 1919, 5, pp. 141–163; *Trop. Dis. Bull.,* 1921, 17, p. 48.

Sweet, W. C. *Fasciolopsis buski* infestation. *Jour. Am. Med. Assoc.,* 1921, 76, pp. 1819–1820.

Cole, A. F. Five cases of Fasciolopsis infection, with remarks. *Trans.* Roy. Soc. Trop. Med. and Hyg., 1921, 14, pp. 93–98; *Trop. Dis. Bull.,* 1922, 19, p. 216.

Muto, M. Ueber den ersten Zwischenwirt des Metagonimus yokogawa (Japanese text). Kyoto Igakw Zassi (*Kioto Jour. Med. Sci.*), 1917, 14, pp. 115–134 (author's summary in German); *Trop. Dis. Bull.,* 1918, 12, p. 175.

MUTO, M. Biologische Studien über die Cercarien und die encystierten Cercarien des *Metagonimus yokogawi* (Japanese text). *Kyoto Igakw Zassi,* 1917, 14, pp. 79–100 (author's summary in German); *Trop. Dis. Bull.,* 1922, 12, pp. 175–176.

MUTO, S. Intermediate host of *Metagonimus yokogawai.* Saikingaku Zasshi (*Jour. Bacteriol.*), No. 258, p. 288; *Trop. Dis. Bull.,* 1918, 12, p. 176.

KOBAYASHI, H. (Life-history of *Clonorchis sinensis*). *Mitteil. a. d. Medz. Faschschule z. Kejjo,* pp. 257–284; *Trop. Dis. Bull.,* 1918, 11, p. 73.

MEBIUS, J. Clonorchiosis hepatis, cirrhosis parasitaria en typische groei van het galgangepitheel. Geneesk. *Tijdschr. v. Nederl. Indie,* 1920, 60, pp. 224–294; *Trop. Dis. Bull.,* 1922, 19, p. 220.

NAKAGAWA, K. The mode of infection in pulmonary distomiasis. Certain fresh-water crabs as intermediary hosts of *Paragonimus westermani* (*ringeri*). *Jour. Inf. Diseases,* 1916, 18, pp. 131–142.

KOBAYASHI, S. On the development of the *Paragonimus westermani* and its prevention. *Japan Med. World., Tokyo,* 1921, 1, pp. 14–17; *Trop. Dis. Bull.,* 1922, 19, pp. 217–218.

MUSGRAVE, W. E., AND CLEGG, M. Paragonimiasis in the Philippine Islands. *Philippine Jour. Sci.,* 1907, Sec. B, 2, pp. 15–63.

KAWAMURA, R., AND YAMAGUCHI, M. On the pulmonary distomiases which caused brain symptoms in children. *Japan Med. World,* 1921, 1, pp. 1–7; *Trop. Dis. Bull.,* 1922, 19, p. 218.

KIMURA, O. A case of cysts in the brain caused by *Paragonimus westermani. Mitt. a. d. Pathologischen Inst. der Kaiserlichen Univ. z. Sendai,* Japan, 1921, 1, pp. 375–384; *Trop. Dis. Bull.,* 1922, 19, pp. 219.

LEIPER, R. T., AND ATKINSON, E. L. Observations on the spread of Asiatic schistosomiasts, etc. *Brit. Med. Jour.,* 1915, Jan. 30, pp. 201–203; *Trop. Dis. Bull.,* 1915, 6, pp. 295–298.

LANNIG, R. H. Schistosomiasis on the Yangtze river, with report of cases. *U. S. Naval Med. Bull.,* 1914, 8, p. 36; *Trop. Dis. Bull.,* 1917, 9, pp. 266–268.

MAN, W. L. Some practical aspects of schistosomiasis as found in the Orient. Preliminary report, *Jour. Am. Med. Assoc.,* 1916, 67, pp. 1366–1368; *Trop. Dis. Bull.,* 1917, 9, pp. 268–269.

LEIPER, R. T. Reports on the results of the bilharzia mission in Egypt. *Jour. Roy. Army Med. Corps,* 1915, 25, pp. 1–55, 147–192, 253–267. Rev. in *Trop. Dis. Bull.,* 1916.

LUTZ, A. Observacoes sobre a evolucao do *Schistosomum mansoni* Nota previa. *Brazil Medico,* 1916, 30, pp. 385–387; *Trop. Dis. Bull.,* 1917, 9, p. 271.

GONZALEZ, E. The intermediary host of *Schistosomum mansoni* in Venezuela. *Nat. Acad. Med. Caracas,* 1917.

FANTHAM, STEPHENS AND THEOBALD. *The Animal Parasites of Man.* Wm. Wood and Co., New York, 1916. (The best textbook on the Trematoda as well as on other worm parasites of man.)

LEIPER, R. T. Medical helminthology: A review. *Trop. Dis. Bull.,* 1922–23, 19, pp. 361–365; 20, pp. 857–864.

Tropical Diseases Bulletin contains reviews of all articles on trematodes published to date in any language.

XI. The Cestoda

Definition.—The tapeworms are ribbon or tape-shaped flat worms divided into sexually complete (hermaphrodite) segments (proglottids) and having an organ of attachment (scolex) at one end of the chain of segments bearing suckers and hooks. They live as parasites in the small intestine of man and other animals.

Classification.—

Subkingdom II:	Metazoa
Phylum VII:	Plathelminthes
Class III:	Cestoda
Order III:	Pseudophyllidea
Family:	Dibothriocephalidae
Genus:	*Dibothriocephalus*
Species:	*D. latus*
Order V:	Cyclophyllidea
Family:	Hymenolepididae
Genus:	*Hymenolepis*
Species:	*H. nana*
Family:	Taeniidae
Genus:	*Taenia*
Species:	*T. saginata*
	T. soleum
	T. echinococcus

Historical.—Read historical introduction to section on the Cestoda in Fantham, Stephens and Theobald (1916).

General morphology.—The tapeworms are a group of animals that have undergone profound anatomical and physiological modifications consequent to their extreme adaptation to parasitism. In general these modifications are an increase in the organs and functions of reproduction and a degeneration of other organs and functions. Apart from the so-called head bearing the organs of parasitic attachment, the tapeworm is a chain of sexually complete (hermaphroditic) segments that are being constantly formed at the attached end and as constantly cast off, filled with ripe ova, at the distal end of the chain. There are rudimentary nervous and excretory systems common to the whole chain, but no alimentary tract or organs of special sense. Study preserved specimens and mounted preparations of tapeworms with special reference to scolex, proglottids, reproductive organs, nerve trunks, and excretory canals.

Read section of anatomy of the cestodes in Fantham, Stephens and Theobald (1916).

General life-history.—The tapeworms, with few exceptions, have an alternation of hosts but no alternation of generations. Compare with Trematoda. In the intestinal tract of the definitive host the adult tapeworm produces ova which pass out with the feces; in the intermediary host, the ingested ova hatch larvae which penetrate the tissues and become encysted. A new definitive host becomes infected by eating the uncooked or insufficiently cooked flesh of the intermediary host containing the encysted larvae.

Read sections on the development and biology of the cestodes *in* Fantham, Stephens and Theobald (1916).

THE MORE IMPORTANT TAPEWORMS OF MAN

1. *Dibothriocephalus latus* (Broad or fish tapeworm)—

Morphology.—Study preserved specimens and mounted preparations of this tapeworm with special reference to scolex, suckers, proglottids, and reproductive organs. Compare structure of reproductive system with that of the Trematoda. Make notes and drawings.

Read section on *Diblothriocephalus latus* in Fantham, Stephens and Theobald (1916).

Life-history.—First intermediary host, certain fresh-water crustaciae (Janicki and Rosen, 1917); second intermediary host, certain fresh-water fish (Fantham, Stephens and Theobald, 1916); definitive hosts, man, dog, cat.

Mode of infection.—Man becomes infected by eating raw or insufficiently cooked fish containing larvae (pleroceroids).

Geographical distribution.—See Fantham, Stephens and Theobald (pp. 313–314).

Pathogenesis.—*Dibothriocephalus latus* lives in the small intestine of man. It may produce a severe anaemia, known as dibothriocephalus anaemia caused by a toxin (Fantham, Stephens and Theobald, pp. 644–646).

Diagnosis.—By microscopic examination of the feces for ova. Study the ova of *D. latus* in preserved feces and note characters. Compare with ova of the Trematoda. Make notes and drawings.

2. *Hymenolepis nana* (Dwarf tapeworm)—

Morphology.—Study preserved specimens and mounted preparations of *H. nana* and compare the structure of the scolex, proglottids, and reproductive organs with those of *Dibothriocephalus latus.* Make notes and drawings.

Read section on *H. nana* in Fantham, Stephens and Theobald (1916).

Life-history.—Not yet elucidated. Compare with life-history of *H. murina* parasitic in the rat, as described by Grassi (Fantham, Stephens and Theobald, pp. 324–325). Read Saeki (1920).

Mode of infection.—Cannot be determined until life-history is known.

Geographical distribution.—Scattered, see Fantham, Stephens and Theobald.

Pathogenesis.—Causes digestive and nervous disorders in children. See Fantham, Stephens and Theobald (1916).

Diagnosis.—By ova in feces. Study ova in preserved feces and determine characters. Make notes and drawings. Read description of ova in Fantham, Stephens and Theobald.

3. *Taenia saginata* (Beef tapeworm)—

Morphology.—Study preserved specimens and mounted preparations of *T. saginata* with special reference to scolex, proglottids, structure of reproductive organs in sexually mature segments and of ripe segments filled with ova. Make notes and drawings. Read section in Fantham, Stephens and Theobald.

Life-history.—The adult worm lives as a parasite in the small intestine of man exclusively. Ova are produced which escape with the feces. When these ova are ingested by the ox or cow, the oncospheres escape from the shell in the intestine, penetrate the tissues, settle in the muscles and develop into the larval stage (cysticercus). This larval stage undergoes no further development until it is taken into the digestive tract of its definitive host, man, when it develops into the adult tapeworm.

Mode of infection.—Man becomes infected by eating raw beef or beef insufficiently cooked to kill the cysticerci.

Geographical distribution.—Widely distributed over the whole world.

Pathogenesis.—Read Fantham, Stephens and Theobald, pp. 667–668.

Diagnosis.—By ova and ripe proglottids in the feces. Study the ova in preserved feces and note their characteristics. Study stained slides of ripe proglottids with special reference to the uterus and its branches. Make notes and drawings. Read description of ova and ripe proglottids in Fantham, Stephens and Theobald.

4. *Taenia solium* (Pork tapeworm)—

Morphology.—Study mounted specimens. Make notes and drawings. Read description of this tapeworm in Fantham, Stephens and Theobald. Compare with *T. saginata* with special reference to scolex and ripe proglottids.

Life-history.—Similar to *T. saginata,* except that the pig is the intermediary host. Read section on life-history in textbooks.

Mode of infection.—Man becomes infected by eating raw or insufficiently cooked pork containing cysticerci.

Geographical distribution.—Distribution is coextensive with domestic pig and the custom of eating raw or insufficiently cooked pork. Read section in textbooks.

Pathogenesis.—Similar to that of *T. saginata* in the case of the adult tapeworm in the intestine. There is, however, a much more serious aspect of infections with *T. solium* than with *T. saginata,* in that persons infected with the former are subject to direct infection with the ova and the development of the cysticerci which may become localized in the eye or brain. Read Fantham, Stephens and Theobald, pp. 662–666.

Diagnosis.—By presence of ova or ripe proglottids in the feces. Study ova and segments of *T. solium.* Compare with *T. saginata.* Read descriptions and study figures of ova and ripe segments of *T. solium* in textbooks and compare with those of *T. saginata* with special reference to a differential diagnosis.

5. *Taenia echinococcus* (Dog tapeworm)—

All of the tapeworms hitherto considered live as sexually mature adults in the intestinal tract of man, that is, man is the definitive host; *Taenia echinococcus* lives as a sexually mature adult worm in the intestinal tract of the dog, and it is the larval or cysticerus stage that occurs in man, that is, man is the intermediary host of this tapeworm.

Morphology.—Read section on *Taenia echinococcus* in Fantham, Stephens, and Theobald. Note small size, few segments, structure of scolex and of proglottids.

Life-history.—Similar to that of *T. saginata* and *T. solium,* except that man (and cattle) is in this case the intermediary host and harbors the cysticercus stage in his tissues and that the cysticercus stage (echinococcus cyst) is a more complex larval stage than that of the other tapeworms. Examine specimens of echinococcus cyst. Read description and examine figures of structure of echinococcus cyst in Fantham, Stephens and Theobald.

Mode of infection.—By ingestion of ova of *Taenia echinococcus* from the dog.

Geographical distribution.—Central Europe, Iceland, Argentina, Paraguay and Australia especially, and occasionally in other countries.

Pathogenesis.—The echinococcus cyst, which may develop in various organs, especially the liver and lungs, and may attain the size of a child's head, is a very dangerous infection. Read section in Fantham, Stephens and Theobald on echinococcus infection.

Diagnosis.—Since it is the larval stage of *T. echinococcus* that infects man and the infection is located in the internal organs, diagnosis is clinical. Beside the ordinary physical diagnosis, 2 serological tests have been employed, namely (1) the precipitin test, and (2) complement deviation (Fantham, Stephens and Theobald, p. 359).

Prophylaxis of cestode infections.—Tapeworms having the larval, infective, stage in fish (*Dibothriocephalus latus*) or in food animals (*Taenia saginata, T. solium*) are transmitted to man by the ingestion of infected flesh of the intermediary host; therefore infection can be avoided by taking care not to eat uncooked or insufficiently cooked fish, beef, or pork in the endemic regions of the respective tapeworms. Government inspection of pork and beef has helped to decrease the spread of these infections. The infection with the cysticercus stage of *T. solium* may be auto-infectious in individuals having the adult tapeworm in their intestine or from others infected with *T. solium*. Preventive measures obviously include expulsion of the adult tapeworms from the intestine of infected individuals, the exclusion of infected persons from handling food, and personal cleanliness. Since echinococcus infections result from the ingestion of ova of the dog tapeworm, the close association with dogs in endemic regions is to be avoided.

References:

Janicki, C., et Rosen, F. Le cycle evolutif du *Dibothriocephalus latus* L. Recherches experimentales et observations, etc. *Bul. Soc. Neuchateloise des Sci. Nat.*, 1917, 42; *Trop. Dis. Bull.*, 1921, 17, pp. 64–66.

Saiki, Y. Experimental studies on the development of Hymenolepis nana T. *Jiki Zasslii* (*Jour. Pediatrics*), 1920, Mar. 20, No. 238, pp. 203–244; *Trop. Dis. Bull.*, 1921, 18, p. 112.

Fantham, Stephens and Theobald. *The Animal Parasites of Man.* Wm. Wood and Co., New York, 1916.

Leiper, R. T. Medical helminthology: A review, Part II. *Trop. Dis. Bull.*, 20, pp. 85–91.

Tropical Diseases Bulletin (London), 1912–23. Review of all current literature on the Cestodes.

XII. The Nematoda

Definition.—Filiform or fusiform, bilaterally symmetrical, unsegmented worms, having a body cavity, complete alimentary canal with mouth and anus and almost invariably separate sexes. They live as parasites in the intestine and other organs, the tissues and the blood of man and other animals.

Classification.—

Subkingdom II:	Metazoa
Phylum VIII:	Coelhelminthes
Class II:	Nemathelminthes
Order I:	Nematoda
Family:	Ascaridae
Genus and species:	*Ascaris lumbricoides*
Family:	Oxyuridae
Genus and species:	*Oxyuris vermicularis*
Family:	Trichinellidae
Genus and species:	*Trichuris trichiura*
	Trichinella spiralis
Family:	Strongylidae
Genus and species:	*Ancylostoma duodenale*
	Necator americana
Family:	Angiostomidae
Genus and species:	*Strongyloides stercoralis*
Family:	Dracunculidae
Genus and species:	*Dracunculus medinensis*
Family:	Filaridae
Genus and species:	*Filaria bancrofti*
	Loa loa
	Onchocercus volvulus

General morphology.—Dissect a male and a female *Ascaris lumbricoides* by opening the body wall longitudinally along a lateral line. Study the anatomy of this worm with special reference to shape, external opening, body wall, body cavity, alimentary canal, and reproductive organs. Make notes and drawings. Read section on the anatomy of the nematodes in Fantham, Stephens and Theobald.

General life-history.—Variable in the different species. See special life-histories under the several species.

Read section on development of the nematodes in Fantham, Stephens and Theobald.

THE MORE IMPORTANT NEMATODE PARASITES OF MAN

1. *Ascaris lumbricoides*—

Historical.—Parasite known since ancient times.

Morphology.—Study specimens of *Ascaris* with special reference to shape, size, color, mouth, anus, genital openings, and sexual dimorphism. Read section in Fantham, Stephens and Theobald.

Life-history.—Ova are produced by the female, after copulation with the male, which pass out of the host with the feces. These ova require a certain period of incubation in the soil, during which an embryo is developed coiled in the ovum, before they become infective. The infection is direct without an intermediary host; young larvae hatched from the ingested ova leave the intestine and wander through the tissues, especially the lungs, before returning to the small intestine to grow into adult ascarids.

Read section on life-history in Fantham, Stephens and Theobald (1916); Stewart (1916–18); Ransom and Foster (1917–20); Yoshida (1919).

Mode of infection.—By ingestion of ova that have developed larvae after sufficient incubation in the soil.

Geographical distribution.—World-wide, and one of the most frequent intestinal worm parasites of man.

Pathogenesis.—The adult worms live normally in the small intestine where, if in large numbers, they may give rise to severe local symptoms of a toxic or reflex nature. More serious symptoms and even death may arise from the migrations of the worms into the bile-ducts, stomach, oesophagus, respiratory passages, and other locations. Read Fantham, Stephens and Theobald (1916), pp. 687–692; Crowell, B. C. (1920). The migrating larvae may cause pulmonary symptoms (Ransome, 1919); Koino (1922).

Diagnosis.—By ova in the stools. Study ova of *Ascaris* in preserved feces with reference to the characters of typical and atypical eggs. The ova of *Ascaris* show great variations from the type. Make notes and drawings.

Prophylaxis.—Proper disposal of feces; avoidance of dirt-eating by children, and contamination of food or water with polluted soil.

2. *Oxyuris vermicularis* (Pinworm)—

Morphology.—Study preserved and mounted specimens of *Oxyuris* with reference to size, shape, dorso-ventral cuticular expansions at the anterior end, and internal anatomy. Make notes and drawings. Read section on *Oxyuris* in Fantham, Stephens and Theobald.

Life-history.—*Oxyuris vermicularis* lives in the lower part of the small intestine and the caecum; the females containing mature ova migrate to the rectum and anus, and even outside the anus, where they cause intolerable itching, and deposit the ova. The ova at the time they are deposited contain a fully developed embryo and are capable of infecting directly without further development. Read section on development in Fantham, Stephens and Theobald.

Mode of infection.—By direct or indirect contagion with fingers, towels, bed linen, etc., contaminated with ova. Auto-reinfection is common, and spread of the infection closely associated among children, as in institutions, almost always occurs.

Geographical distribution.—World-wide, infecting children especially.

Pathogenesis.—The effect of the worms on the host is chiefly one of irritation. When present in large numbers they have been accused of causing enterocolitis and appendicitis.

Read Fantham, Stephens and Theobald (1916), pp. 694–696.

Diagnosis.—By the ova in the feces. Study ova of *Oxyuris* in preserved feces and note characteristics. Make notes and drawings. Compare with ova of hookworms.

Prophylaxis.—As infection is by direct or indirect contact, prophylactic measures are in general those with other intestinal contagious diseases, with special reference to soiled fingers, underwear, towels, bed linen, etc. Auto-reinfection in children is common from fingers, which have become contaminated in attempts to allay the intolerable itching caused by the worms.

3. *Trichuris trichiura*—

Morphology.—Study mounted slide of *Trichuris*. Make notes and drawings.

Read section on morphology in Fantham, Stephens and Theobald.

Life-history.—The adult male and female worms live in the caecum of man. The ova pass out with the feces and require a certain period for the development of an intra-ovum larva in water or soil before they become infective. Read section on development in Fantham, Stephens and Theobald.

Mode of infection.—Infection is caused by the ingestion of matured ova containing larvae.

Geographical distribution.—World-wide.

Pathogenesis.—Read Fantham, Stephens and Theobald (1916), pp. 678–680; Musgrave and Clegg (1908).

Diagnosis.—By ova in feces. Study ova in preserved feces. Make notes and drawings.

Prophylaxis.—Proper disposal of feces; avoidance of fecal contamination of food and water.

4. *Trichinella spiralis*—

Morphology and life-history.—*Trichinella spiralis* is a small nematode that lives in the adult stage in small intestine of the rat, pig, and man. The ova hatch in the intestine and do not leave the body of the host, but penetrate the tissues and become encysted in the muscles.

Read section on morphology and development of *Trichinella spiralis* in Fantham, Stephens and Theobald. Study sections of muscle containing encysted *Trichinella.* Make notes and drawings.

Mode of infection.—The rat is the normal host of *Trichinella* and the infection is transmitted from rat to rat by the cannibalistic habits of this rodent. The pig becomes infected by eating the dead bodies of infected rats. Man becomes infected by eating uncooked or insufficiently cooked pork containing the encysted larvae of *Trichinella.*

Geographical distribution.—More or less world-wide in rats and pigs; in man infections depend to a large degree on the habit of eating uncooked or insufficiently cooked pork, as is especially the case in some parts of Europe. (See Fantham, Stephens and Theobald, 1916, pp. 427–429.)

Pathogenesis.—*Trichinella spiralis* causes a serious and often fatal disease known as trichinosis. Read section on symptomatology and pathology in Fantham, Stephens and Theobald, pp. 424–427; 680–681.

Prophylaxis.—Government inspection of pork; thorough cooking of a pork product. Read Fantham, Stephens and Theobald, pp. 429–431.

5. Hookworms: *Ancylostoma duodenale; Necator americana.*

Morphology.—Study mounted slide of male and female *Ancylostoma duodenale* with special reference to shape, size, head, tail, and structure of reproductive organs. Make notes and drawings. Examine preserved specimens of hookworms, select specimen of *Necator americana* and compare morphology with that of *A. duodenale,* with special reference to structure of mouth capsule and of the bursa of the male. Make notes and drawings. Read section on morphology of *A. duodenale* and *N. americana* in Fantham, Stephens and Theobald.

Life-history.—The adult male and female hookworms live in the small intestine of man. The ova are deposited in the intestine and pass out with the feces. In the soil the ova hatch small larvae which

undergo growth, development and several moults. At the last moult the larva remains enclosed in the old skin (for protection) and is now in the infective stage. This infective larva is actively motile and will live for some time under favorable conditions, but without further development until it has entered its host (man). Examine mounted slide of hookworm larvae; note characters; made notes and drawings. Read section on development of the hookworm in Fantham, Stephens and Theobald.

Mode of infection.—The infective larvae of the hookworms penetrate the skin of persons coming in contact with infested soil. The larvae follow a roundabout course through the blood, lungs, larynx, oesophagus to the small intestine (Loos, 1899–1905). Infection may also follow the ingestion of larvae with contaminated food and water. Read section in Fantham, Stephens and Theobald on mode of infection.

Geographical distribution.—Ancylostoma duodenale was originally a European and Asiatic parasite, while *Necator americana* was an African species brought to America by the Negro. Both species are now more or less intermingled and of world-wide distribution.

Pathogenesis.—During the penetration of the skin, the larvae may, if in large numbers, set up local irritation known as "ground-itch." The adult hookworms live in the small intestine, especially in the jejunum, of man. They attach themselves by the buccal capsule to the intestinal mucosa and feed upon it. The symptomatology and pathology depends largely on the degree of infection. A few hookworms will produce no obvious symptoms; typical hookworm disease results from massive infections or upon repeated reinfections. Progressive anemia and fatty degeneration of the viscera are the most prominent effects of heavy infections. The infections are widespread in the tropics and subtropics and, in many regions, are an important cause of morbidity.

Read sections on symptomatology and pathology in Castellani and Chalmers; Manson-Bahr; Stitt; Fantham, Stephens and Theobald, pp. 682–684.

Prophylaxis.—Proper disposal of feces; treatment of infected persons; protection of feet from polluted soil.

Read Cort and collaborators (1921–1922); Smillie (1922); Darling (1922); and other publications of investigations conducted by the International Health Board.

6. *Strongyloides stercoralis*—

Historical.—Read historical sketch in Fantham, Stephens and Theobald.

Morphology.—Study mounted specimens of *S. stercoralis* with special reference to shape, size, alimentary tract, and uterus filled with ova. Make notes and drawings. This worm reproduced parthenogenetically (or hermaphroditically) and there is no male (the only exception to the sexual differentiation among the nematode parasites of man). Read section on morphology in Fantham, Stephens and Theobald.

Life-history.—The life-history of this parasite is complex, there being a parasitic and a free-living generation. The parasitic worm produces ova parthenogetically or hermaphroditically which hatch in the intestine of man, and the rhabditiform larvae pass out of the host with the feces. These rhabditiform larvae develop into males and females of the free-living generation. The males and females copulate and the females produce ova that hatch in the soil, producing a free generation of rhabditiform larvae. These larva grow, moult, and develop into filariform larvae which, enclosed in the last moulted cuticle, constitute the infective stage for man. This life-history holds true for *S. stercoralis* in the tropic. In cold climates the free-living generation is omitted and the rhabditiform larvae of the parasitic generation develop directly into the infective filariform larvae. Study mounted slides of the different stages of the life-history of *S. stercoralis* and connect them with the life-cycle of the worm. Make notes and drawings. Read section on the life-history of *S. stercoralis* in Fantham, Stephens and Theobald.

Mode of infection.—Similar to the mode of infection with hookworms. The filariform larvae penetrate the skin of persons walking barefoot in infected soil, and make their may to the blood stream, by which they are carried to the lungs. In the lungs they leave the blood vessels and enter the bronchi, make their way up to the oesophagus and down the intestinal tract, where they settle in the small intestine. Infection can also result from ingestion of food or water contaminated with the filariform larvae. Read Van Durm (1902); Fulleborn and Schilling-Torgan (1911).

Geographical distribution.—More or less world-wide, but more prevalent in the tropics. Everywhere the incidence of infection is less than would be expected considering the multiplication of the infective larvae in the soil. Reason for this?

Pathogenesis.—*Strongyloides stercoralis* lives in the small intestine where it burrows deep into mucosa. When present in large numbers its may cause intestinal catarrh leading to anemia and intermittent diarrhoea.

Diagnosis.—*Strongyloides stercoralis* is the only intestinal worm parasite that is not diagnosed by the ova in the feces; since the ova hatch before leaving the body of the host, the diagnosis is made by

the presence of motile rhabditiform larvae in the stools. Study mounted specimens of the rhabditiform larvae, noting its characters, especially the double swelling of the oesophagus. Make notes and drawings. What is a possible source of error in diagnosis?

Prophylaxis.—Proper disposal of feces; avoid skin contact with infested soil and fecal contamination of food and water.

7. *Dracunculus medinensis* (Guinea worm)—

The Guinea worm lives, in the adult stage, in the subcutaneous tissues, especially of the lower extremities, of man. The viviparous larvae are discharged through local abscesses into water. Intermediary host a small fresh-water crustacean of the genus, *Cyclops*. Infection of man probably by the ingestion of infected cyclops with water.

Africa, Arabia, Near East, India. Read section in Fantham, Stephens and Theobald.

8. *Filaria bancrofti*—

Definition.—The filariidae are long, slender, hair-like nematodes living as adults in the serous cavities, lymphatic glands, or subcutaneous tissues of man and other animals. They are viviparous and the larvae are found in the circulating blood or lymph and are transmitted by blood-sucking insects. *Filaria bancrofti* is the most common and widely distributed filaria of man, the larvae of which appear in the peripheral circulation at night (*Filaria nocturna*).

Historical.—Read historical sketch in Fantham, Stephens and Theobald (1916), pp. 390–391.

Morphology.—Examine preserved specimens of *Onochocerca volvulus* for general morphology of the Filaridae. Read section in Fantham, Stephens and Theobald on the morphology of *Filaria bancrofti* and compare with that of *O. volvulus*.

Life-history.—*Filaria bancrofti* lives in the adult stage in the lymphatic glands and vessels of man. The larvae, which are produced viviparously by the female, are found in the circulating blood. They show a well-marked periodicity in the peripheral circulation, appearing in appreciable numbers only at night. These circulating larvae, when taken up with the blood by a culecine mosquito, undergo a certain development in the stomach and muscles, and finally reach the labrium of the mosquito, as first demonstrated by Manson (1884). They are inoculated into man by the bites of these infected mosquitoes.

Read section on the life-history in Fantham, Stephens and Theobald (1916); Manson, P. (1884).

Mode of infection.—Man becomes infected by the bites of mosquitoes infected with the larvae of *Filaria bancrofti*.

Geographical distribution.—More or less general in the tropics and subtropics. The infection occurs in the southern United States.

Pathogenesis.—Filaria bancrofti infections are associated with lymphangitis, varicose glands, chyluria, chylocele, lymph scrotum, orchitis, abscess, and elephantiasis.

Read section on pathology and symptomatology in Fantham, Stephens and Theobald (1916); Bahr (1912, 1914); Castellani and Chalmers.

Diagnosis.—

I. Laboratory.

By presence of larvae in the blood. Preparations should be made from blood taken at night. They may be examined fresh for motile larvae or as thick smear preparations, dehemoglobinized and stained. Examine stained preparations for larvae of *Filaria bancrofti.* Make notes and drawings of larvae, their relative size as compared with red blood corpuscles, and general characters.

II. Clinical.

Read section on symptomatology of filarial infections in Fantham, Stephens and Theobald; Castellani and Chalmers; Manson-Bahr.

Prophylaxis.—Protection from bites of mosquitoes in endemic regions; mosquito control.

9. Other *Filariae* of man.

(*a*) *Loa loa.*—The adults live in the subcutaneous connective tissue, frequently of the conjunctiva and in the anterior chamber of the eye. The larvae have, in contrast to those of *Filaria bancrofti,* a diurnal periodicity (Filaria diurna). This filaria is found in West Africa and is transmitted by a blood-sucking fly of the genus *Chrysops.* Read section in Fantham, Stephens and Theobald.

(*b*) *Acanthocheilonema perstans.*—The adults inhabit the connective tissue at the base of the mesentery. The larvae have no definite periodicity, but are found in the peripheral circulation both day and night (*Filaria perstans*). This parasite occurs in Africa and its mode of transmission is unknown. Read section in Fantham, Stephens and Theobald.

(*c*) *Onchocerca volvulus.*—The adults live in subcutaneous tumors. The larvae appear to live in the lymphatics rather than the blood. This species is found in West Africa, and a variety of it occurs on the west coast of Central America and causes ocular symptoms (Pacheco-Luna, 1920). Life-history unknown.

References:

FANTHAM, STEPHENS AND THEOBALD. *The Animal Parasites of Man.* Wm. Wood and Co., 1916. (This is the best general textbook on the nematodes.)

STEWART, F. H. On the life-history of *Ascaris lumbricoides. Brit. Med. Jour.* 1916, 2, July, pp. 5–7; *ibid.*, Oct. 7, pp. 486–488; *ibid.*, Dec. 2, pp. 753–754; *Parasitology,* 1917, 9, pp. 213–227.

RANSOM, B. H., AND FOSTER, W. D. Life-history of *Ascaris lumbricoides* and related forms. *Jour. Agri. Research,* 1917, 11, 8, pp. 395–398; *Jour. Parasitology,* 1919, 5, pp. 93–99; *U. S. Dept. Agr.,* Bull. 817, 1920.

YOSHIDA, S. On the migrating course of ascarid larvae in the body of the host. *Jour. Parasitology,* 1919, 6, pp. 19–27.

RANSOM, B. H. A newly recognized cause of pulmonary disease, *Ascaris lumbricoides. Jour. Am. Med. Assoc.,* 1919, 73, Oct. 18, pp. 1210–1212.

CROWELL, B. C. The dangers of ascariases. *Am. Jour. Med. Sciences,* 1920, 99, pp. 380–398.

KINO, S. Tomyo-Iji-Shinshi (*Tokyo Med. News*), Oct., 1922, No. 2, p. 229; Rev. in *Trop. Dis. Bull.,* 1923, 20, pp. 636.

MUSGRAVE, W. E., AND CLEGG, M. T. Trichocephaliasis. *Philippine Jour. Sci. B. Med. Sci.,* 1908, 3, pp. 545–566.

LOOSS, A. Über des Eindringen der Ancylostomalarven in die menschliche Haut. *Centralbl. f. Bakteriol.,* etc., 1 Abt., 1900?, 29, p. 733.

LOOSS, A. Die Wanderung der Ancylostomun und Strongyloides-Larven von Haut nach dem Darm. C. R. 6e Congr. mit. de Zool. 1905. (Berne, 1904), p. 225.

STILES, C. W. New species of hookworm (Uncinaria (Nector) americana) parasitic in man. *Am. Med.,* 1902, 3, p. 777.

CORT, W. W., and collaborators. Investigations on the control of hookworm disease. *Am. Jour. Hyg.,* 1921, pp. 5–6, 557–568; *ibid.,* 1922, 2, pp. 1–50, and following numbers.

DARLING, S. T. The hookworm index and mass treatment. *Am. Jour. Trop. Med.,* 1922, 2, pp. 397–447.

SMILLIE, W. G. The results of hookworm disease prophylaxis in Brazil. *Am. Jour. Hyg.,* 1922, 2, pp. 77–95.

International Health Board, The Rockefeller Foundation. *Bibliography of hookworm disease.* Publ. No. 11, 1922.

VAN DURME, P. Quelques notes sur les embryons de Strongyloides intestinalis et leur penetration par la peau. *The Thompson Yates Lab. Rept.,* 1902, 2, pt. 2, p. 471.

FULLEBORN, F., UND SCHILLING-TORGAN, V. Untersuchungen über den Infektionsweg bei Strongyloides und Ankylostomum. *Arch. f. Schiffs.- u. Tropen-Hyg.,* 1911, 15, pp. 569–571.

MANSON, P. The metamorphosis of Filaria sangunis hominis in the mosquito. *Trans. Linn. Soc.,* London, 1884, 2, pp. 10, 367.

BAHR, P. H. Filariasis and elephantiasis in Fiji. *Jour. London School Trop. Med.,* 1912, Suppl. 1; also *Trans.* Soc. Trop. Med .and Hyg., 5, pp. 131–155; also published in book form. London, Witherby and Co.

STENHOUSE, H. M. Real cause of elephantiasis. *U. S. Naval Med. Bull.,* 1925, 22, pp. 119–225.

Tropical Diseases Bulletin, London. Reviews of all literature on the nematodes to date.

APPENDIX A

PREPARATION AND MODE OF APPLICATION OF SOLUTIONS AND STAINS— BIOCHEMICAL TESTS

PREPARED BY

P. SCHOENHOLZ

APPENDIX A

PREPARATION AND MODE OF APPLICATION OF SOLUTIONS AND STAINS—BIOCHEMICAL TESTS

PREPARED BY

P. SCHOENHOLZ

Solutions and Stains

Absolute alcohol.—Absolute commercial alcohol always contains from ½ to 1 per cent water. It may be eliminated by adding dehydrated copper sulphate. The copper sulphate is prepared in the following way: Burn ordinary copper sulphate in a porcelain, or preferably copper, dish until it is white. Carefully wrap the white powder in filter paper and place it in the bottle containing the absolute alcohol.

To prepare the more dilute solutions of alcohol, the following proportions of alcohol and distilled water are mixed:

	C.c. distilled water	C.c. 95 per cent alcohol
90 per cent alcohol	32.5	500
80 per cent alcohol	80.6	400
70 per cent alcohol	156.6	400
60 per cent alcohol	189.5	300
50 per cent alcohol	289.0	300

Acetone alcohol.—To 70 c.c. of either absolute alcohol or 95 per cent alcohol, add 30 c.c. of acetone.

Use: For decolorization in the Gram staining method.

Acid alcohol.—To 97 c.c. of 70 per cent alcohol, add 3 c.c. of concentrated hydrochloric acid.

Use: In the demonstration of acid fast bacteria.

Acid mercuric chloride.—

$HgCl_2$	0.5 gms.
$NaHSO_4$ (Sodium bisulphate)	1.1 gms.
Tap water	1000.0 c. c.

Use: As a disinfectant for hands.

Alcohol-ether.—A solution composed of equal parts of absolute alcohol and ether may be used to remove fats from microscopic preparations previous to staining.

Ammonium oxalate solution.

Ammonium oxalate	0.2 gms.
Sodium chloride	0.6 gms.
Distilled water	100.0 c. c.

Use: In taking blood cultures to prevent coagulation of the blood.

Ammonium oxalate gentian violet.—

Gentian or crystal violet (sat. alc. sol.)	10 c. c.
Ammonium oxlate (1 per cent aqueous sol.)	40 c. c.

Use: In Gram stain (Hucker and Conn's modification).

Andrade's indicator.—100 c.c. of 0.5 per cent solution of acid fuchsin (National Aniline Company) is decolorized by the addition of approximately 16 c.c. of a normal solution of sodium hydroxide. The color of the solution is restored by acids. The alkali should be added very slowly and the mixture should be shaken well between each addition of alkali. The color of the fuchsin will gradually change from red to wine and then to a straw color which is the end reaction.

Use: As an acid indicator in culture media. Turns red when the reaction becomes acid.

Anilin water.—Add 5 c.c. of anilin oil to 125 c.c. of distilled water. Shake thoroughly, then filter through a double layer of filter paper well moistened with distilled water to retain the undissolved anilin.

Use: Preparation of anilin—water gentian violet.

Bismark brown.—Add 10 c.c. of saturated filtered stock solution of the dye to 90 c.c. of distilled water.

Use: As a counter stain. Apply one minute with Neisser's or Gram's methods.

Carbolic acid solution (5 per cent).—Add 5 grams of chemically pure carbolic acid to 100 c.c. of distilled water. Shake until dissolved; or add 6 c.c. of liquid carbolic acid to 90 c.c. of distilled water. Filter through paper.

Use: Preparation of carbolfuchsin.

Concentrated stock staining solutions.—In general the anilin dyes commonly used for the staining of bacteria are made up in concentrated or saturated solutions, either in distilled water or in 95 per cent alcohol. Dilutions are made from such 'stock solutions' after filtration for use in routine staining methods. The strength of various saturated solutions are as follows:

	Saturation strengths	
	In water per cent	In 95 per cent alcohol per cent
Bismark brown	0.5	....
Fuchsin (basic)	1.5	3.0
Gentian violet	1.5	4.8
Methylene blue	6.7	7.0
Methyl violet	....	7.0
Safranin	3.0	....
Thionin in 50 per cent alcohol	....	1.0

The dyes are dissolved in the solvent, shaken, allowed to stand over night, and filtered through filter paper.

Copper sulphate solution.—Dissolve 20 grams of chemically pure copper sulphate by shaking in about 75 c.c. of distilled water, then bring the volume to 100 c.c. with water.

Use: In Hiss capsule stain.

Fuchsin (basic) aqueous.—Add 5 c.c. of filtered alcoholic saturated stock solution of basic fuchsin to 95 c.c. of distilled water.

Use: As a counter stain, apply one-half to one minute.

Fuchsin carbol (Ziehl's solution).—Add 10–20 c.c. of filtered alcoholic stock solution of basic fuchsin to 80–90 c.c. of 5 per cent aqueous solution of chemically pure carbolic acid.

Use: As a spore stain and in demonstrating acid-fast bacilli. In a dilution of 1 part of stain to 9 parts of distilled water the carbol fuchsin may be used as a simple stain or as a counter stain in Gram's method.

Gentian violet—aqueous.—Add 5 c.c. of filtered alcoholic stock solution of gentian violet to 95 c.c. of distilled water.

Use: In Hiss capsule stain.

Gentian violet—anilin water.—Add 14 c.c. of saturated alcoholic solution of gentian violet to 126 c.c. freshly prepared anilin water. Filter. This stain should be freshly prepared every 3 to 4 days.

Use: Principally in Gram's staining method.

Gentian violet—carbol.—Add 9 c.c. of a saturated alcoholic solution of gentian violet to 100 c.c. of a 5 per cent solution of carbolic acid.

Use: Principally in Gram's staining method.

Iodine solution—Lugol's—Mix in a mortar, 1 gram of iodine, 2 grams of potassium iodide, and 5 c.c. of distilled water. Wash this into a bottle and make up to 300 c.c. with distilled water.

Use: Principally in Gram's staining method.

Lacto-phenol.—

Phenol C. P.	10 c. c.
Lactic acid	10 c. c.
Glycerin	20 c. c.
Distilled water	20 c. c.

Keep in dark bottle to preserve the mixture in the colorless form.

Use: To demonstrate and preserve molds and yeasts.

Liquor cresolis compositus.—

Cresylic acid	500 gms.
Linseed oil	300 gms.
NaOH	54 gms.
Alcohol (ethyl)	30 c. c.
Water to make	1000 c. c.

Heat the oil in a vessel over a water bath at 70° C. Dissolve the NaOH in 50 c.c. water, warm to 70° C., and add to the oil. Mix thoroughly, add the alcohol, and continue heating until, by testing, a small portion is found soluble in boiling water without the separation of an oily layer. While yet warm add the cresylic acid and mix thoroughly. Keep at 70° C. until a clear solution is produced. Finally add the remainder of the water to make a total volume of 1000 c.c.

This solution can be used in a 1–2 per cent solution as a hand wash, and in a 5 per cent solution for general disinfection purposes.

Methylene blue (Loeffler's alkaline).—Add 30 c.c. of a saturated alcoholic solution of methylene blue to 100 c.c. of a 0.01 per cent potassium hydrate solution (1 c.c. of a 1 per cent solution in 99 c.c. of distilled water gives a 0.01 per cent solution).

Use: As a simple stain and for the differentiation of *C. diphtheriae* in smears.

Methyl red.—

Methyl red	0.1 gm.
Alcohol, 95 per cent	300.0 c. c.

Dilute to 500 c.c. with distilled water.

Use: As an indicator to demonstrate the approximate H-ion concentration of glucose broth cultures, particularly in differentiating *B. coli* of fecal origin from those of other sources. At or below a P_H of 5.0 the indicator is deep rose; about 5.0 it is pale rose to yellow.

Methyl violet—carbol.—To 98 c.c. of distilled water add 2 c.c. of concentrated alcoholic methyl violet 5 B. or 6 B. N. This stain keeps for an indefinite period.

Use: Without diluting, principally in Gram's method of staining.

Mercuric bichloride.—Saturated solution at 20° C. contains 7.4 grams per 100 c.c. water.

$HgCl_2$	8 gms.
Water	100 c. c.

Heat the water, then add the $HgCl_2$; shake until the crystals are dissolved and allowed to cool.

Mercuric bichloride for disinfection.—

Mercuric bichloride	0.5 gms.
Sodium bisulphate	1.1 gms.
Tap water	1000.0 c. c.

Reference:

TOACHIMOGLU, A. Eine Methode zur Verstärkung d. antiseptischen Wirkung d. Sublimates. *Med. Klinik*, 1924, 20, p. 14.

Neutral red solution.—

Neutral red	0.2 gms.
Distilled water	100 c. c.

Use: As an indicator to demonstrate the reducing action of bacteria. Reducing bacteria produce a greenish yellow fluroescence. In the presence of alkali the dye turns yellow.

Neutral red counterstain.—

Neutral red	1 gm.
1 per cent acetic acid	2 c. c.
Distilled water	1000 c. c.

Use: As a counterstain in Gram's method.

Saline (buffered salt solution).—

Sodium chloride	7.0 gms.
Secondary sodium phosphate Na_2HPO_4 (anhydrous)	1.356 gms.
Secondary sodium phosphate ($Na_2HPO_4 + H_2O$)	1.75 gms.
Primary potassium phosphate (KH_2PO_4)	0.2 gms.
Distilled water to make	1000.0 c. c.

Reference:

MASON, C. C., AND SANFORD. *Jour. of Lab. and Clin. Med.*, 1924, 9, p. 313.

Saline (physiological salt solution).—

Physiological salt solution No. 1.—To distilled water (test P_H) add 0.85 per cent pure sodium chloride. Sterilize in the autoclave at 15 lbs. pressure for 20 minutes.

Physiological salt solution No. 2.—To distilled water add 0.9 per cent pure sodium chloride. Prepare just before using.

Use: Washing of sheep corpuscles, preparation of corpuscle suspension, and in the dilution of active substances in connection with the Wassermann reaction, etc. It need not be sterilized for this purpose.

Sodium citrate solution.—

Sodium citrate solution No. 1.—Dissolve 1 gram of chemically pure sodium citrate and 0.85 grams of chemically pure sodium chloride in 100 c.c. of distilled water. Filter; place in tubes; plug tightly with cotton; sterilize in the autoclave at 15 lbs. pressure for 20 minutes.

Use: To prevent clotting in the collection of small amounts of blood for blood cells.

Sodium citrate solution No. 2.—Dissolve 10 grams of chemically pure sodium citrate and 0.85 grams of chemically pure sodium chloride in 100 c.c. of distilled water. Filter; place in tubes, and sterilize as above.

Use: To prevent coagulation of freshly drawn blood, add 1 part of citrate solution to 9 parts of whole blood. Mix well.

Sterling's gentian violet.—Grind 5 grams of gentian violet in a mortar with 10 c.c. of 95 per cent alcohol. After practical solution, add 2 c.c. of aniline oil and then 88 c.c. of distilled water. The grinding is continued a short time and then the mixture is let stand a day or two, when it is filtered through paper. It has the merit of staining quickly and intensely and of keeping many months.

Use: Principally in Gram's method of staining bacteria.

Tincture of iodine.—

Iodine crystals	10 gms.
Potassium iodide	10 gms.
Water	135 c. c.
Ethyl alcohol (95 per cent)	365 c. c.

Rub up the iodine crystals and the potassium iodide in a mortar, then add the distilled water and finally the alcohol.

Use: As a pantherapeutic disinfectant.

Toluidine blue.—

Toluidine blue	0.25 gm.
Acetic acid	2.0 c. c.
Absolute alcohol	5.0 c. c.
Distilled water to make	100.0 c. c.

Use: For the demonstration of polar bodies and in diphtheria diagnosis. Does not overstain.

Thionin—carbol.—Ten c.c. of a saturated solution of thionin in 50 per cent alcohol, are added to 100 c.c. of a 2 per cent solution of carbolic acid.

Use: As a simple stain.

Demonstration of Acid-Fast Bacteria*

I. *Method of Ziehl Neelsen.*—

(1). Apply carbol fuchsin with general steaming for 3–5 minutes.

(2) Wash in water.

(3) Decolorize in 70 per cent alcohol containing 3 per cent of hydrochloric acid (acid alcohol), until only a suggestion of pink remains—almost white.

(4) Wash in water.

(5) Counterstain with Loeffler's methylene blue for one minute.

(6) Wash in water.

(7) Dry between filter papers.

Reference:

Deutsch. Med. Wchnschr., 1882, p. 45; *ibid.*, 1883, p. 247. *Centralbl. f. Bakteriol.*, 1883, p. 600.

II. *Konrich's method.*—

(1) Flood smear with carbol fuchsin.

(2) Steam gently for 3 to 5 minutes or stain without heating for 15 minutes.

(3) Wash in water.

(4) Decolorize with a freshly prepared 10 per cent solution of sodium bisulfite for 1–2 minutes.

(5) Wash in water.

(6) Counterstain for 15–30 seconds with an aqueous solution of malachite green, 50 parts to 100 parts distilled water. The bacilli are red on a green background.

Note.—It is essential that the sodium bisulfite solution be freshly prepared, since it is readily oxidized to the sulfate form.

Reference:

Deutsch. Med. Wchnschr., 1920, 46, p. 741.

* For methods of demonstrating acid-fast bacteria in tissue sections see page 78.

III. *Pappenheim's method of differentiating between the tubercle and the smegma bacillus.*—

Films are fixed by heat in the usual way. Stain with hot carbol-fuchsin 2 minutes. Decolorize by pouring over the slide 4 to 5 times the following solution:

Rosalic acid	1 gm.
Absolute alcohol	100 c. c.
Methylene blue to saturation then add glycerin	20 c. c.

The film is then washed in water and dried. The smegma bacilli are decolorized, the tubercle bacilli are red.

Demonstration of Bacteria by Negative Staining

I. *Benian's Congo red negative method.*—A few drops of a 2 per cent Congo-red solution (filtered) are mixed with a drop of the material suspected of containing spirochaetes and spread over a clean slide to form a film. The slide after being air dried is immersed in a jar of absolute alcohol containing 1 per cent hydrochloric acid. In a few minutes the red color of the film turns to a bluish tint. The slide is removed from the acid alcohol and air dried.

II. *Burri's India ink method* of demonstrating bacteria.*—Place a drop of the material to be examined on a slide. Add 1 drop of India ink and mix quickly. Spread in a thin film over the slide. Examine with oil immersion. The organisms appear as unstained bodies on a black background.

Note.—If the material contains much débris or many cellular elements it will be necessary to dilute the ink with water (1 to 4).

III. *Eisenberg's cyanochin method of demonstrating bacteria, spirilla, etc.*—Cyanochin is a mixture of a saturated solution of china blue and cyanosin. A suspension of the material to be examined is mixed with a few drops of the dye and spread in a thin film over the slide. Allow to dry and examine under high dry and oil immersion. The bacteria appear as white bodies on a bluish violet background. According to Eisenberg the Gram positive organisms may take on a pale rose color while the Gram negative organisms remain colorless.

Reference:

Eisenberg. Undersuchungen über halbspezifische Desinfektionsvorgange. *Centralbl. f. Bakteriol.*, 1 Abt. Orig. 1913, 71, p. 420.

* Nigrosine gives better results than India ink.

Capsule Stain

I. *Method of Hiss.*—

(1) Preparations are made by smearing the organisms in a drop of animal serum, preferably beef blood serum.

(2) Dry in air and fix by heat. (Better preparations can sometimes be made by omitting heat fixation.)

(3) Stain for a few seconds with saturated alcoholic solution of carbol fuchsin. The preparation is flooded with the dye and held for a second over a free flame until it steams.

(4) Wash off dye with 20 per cent aqueous copper sulphate solution. Blot—do not wash.

(5) Dry and mount. The capsule appears as a faint blue halo around a dark red or purple cell body. Better results may frequently be obtained by omitting heat fixation and by washing off the dye with the copper sulphate solution as soon as it begins to steam. Water should not be applied at any stage of the procedure.

II. *Muir's method.*—

(1) Prepare thin film, dry and stain in carbol fuchsin ½ minute, the preparation being gently heated.

(2) Wash slightly in 95 per cent alcohol, then wash thoroughly in water.

(3) Flood preparation in mordant for 5 to 10 seconds.

Mordant

Saturated aqueous sol. mercuric chloride	2 parts
Tannic acid (20 per cent aqueous sol.)	2 parts
Saturated aqueous sol. potash alum	5 parts

(4) Wash in water thoroughly.

(5) Treat with 95 per cent alcohol for one minute. (The preparation should have a pale red color.)

(6) Wash well in water.

(7) Counterstain with methylene blue ½ minute.

(8) Dehydrate in alcohol. Clear in xylol and mount.

Flagella Stains

I. *Plimmer and Paine's method.*—Prepare the stain in the following manner:

Tannic acid c. p.	10 gms.
Aluminum chloride (hydrated)	18 gms.
Zinc chloride	10 gms.
Rosaniline hydrochloride	1.5 gms.
Alcohol, 60 per cent	40 c. c.

The solids are placed together in a mortar, and at once (i.e., before deliquescence) triturated with the alcohol. Ten c.c. of alcohol are used first and the mass is mixed thoroughly, care being taken to mash up the whole of the zinc chloride—at this stage a homogenous paste of golden brown color is obtained—the rest of the alcohol is then stirred in slowly, when the mass goes gradually into a viscous solution of deep red color. In this state the stain appears to remain stable for several years. For use it is diluted with water when nearly complete precipitation occurs, a small amount remaining in solution, evidently as a balanced colloid, which, when applied to the bacterial film, becomes adsorbed upon the flagella and periphery of the organism. No further treatment is necessary, but it is advisable to stain the body of the organisms with fuchsin or methylene blue.

Method of application of the stain.—

(1) The culture must be of suitable age; eighteen to twenty-hour cultures grown at 20° C. usually prove satisfactory. The growth is removed and, with as little disturbance as possible, stirred into water to give a distinctly turbid suspension.

(2) As in all methods for staining flagella, it is essential that the slides be scrupulously clean. Cleaning is best done with chromic acid, and the last traces of grease are got rid of by roasting the slides strongly on a wire gauze over a bunsen.

(3) The slide is allowed to cool to about blood heat, and a drop of the suspension is placed at one end of the slide with a 3 mm. loop. The slide is at once tilted so that the drop runs down the slide. It seems to be very important that the film should dry quickly, hence the use of the warm slide. As an alternative method, the drop may be drawn across the slide by means of a strip of gutta-percha tissue. Some excellent preparations have been obtained in this way.

(4) The mordant is applied to the film without fixing, as follows: 1 part of the stain, 0.5 c.c., is mixed with 4 parts of water, 2.0 c.c., in

a small corked specimen tube. After inverting the tube 3 or 4 times it is allowed to stand for 60 seconds, then filtered through a small filter on to the slide and again allowed to remain for 60 seconds, when a slight bronzing should be visible on the surface. It is then washed rapidly under the tap. (A certain amount of license with the time, up to 2 or even 3 minutes, may not be disastrous, but it is better to adhere rigidly to the times as given.)

(5) The film is then flooded with cold carbol fuchsin for 5 minutes, washed, dried, and examined in oil. If satisfactory, the film may be mounted in Canada balsam or better, in euparal, and no fading of the stain seems to occur.

Reference:

PLIMMER, H. G., AND PAINE, S. G. A new method for the staining of bacterial flagella. *Jour. Path. and Bacteriol.*, 1924, 21, p. 286.

II. *Shunk's method.*—To stain flagella it is absolutely essential that (*a*) the slides and cover slips used should be fat free, (*b*) that the culture be handled gently so as to keep the flagella intact. The cover slips should be cleaned according to the method described on page 12. They should, furthermore, never be handled during the staining process, excepting at the edge.

Cultures are best prepared by growing them for 12–16 hours on an agar slant; then gently emulsify a loopful in about 2 c.c. of sterile tap water. It is best to have the tap water at the incubation temperature of the culture. Incubate the suspension for about 20 minutes. Transfer a drop to a cleaned slide or cover slip—do not attempt to spread the drop over the slide. Allow the drop to dry. Fix for 1½ minutes with *either* of the two following *fixatives:*

No. 1.—

Commercial formalin	1 part
Distilled water	1 part

No. 2.—

5 per cent aqueous chromic acid	2 parts
10 per cent acetic acid	1 part

Wash with water.

MORDANT

Solution A.—

Ferric chloride (1–20 aqueous solution)	1 part
Saturated aqueous solution tannic acid	3 parts

This solution improves with age and should be at least a week or two old. It should, therefore, be kept made up in stock solution. When it is being used at frequent intervals, filtering every few days will suffice.

Solution B.—

Aniline oil	1 part
95 per cent alcohol	4 parts

Apply the mordant; 8 drops of solution A followed immediately by 1 drop of solution B. Allow the combination to act at room temperature for 2 minutes. Wash with water and drain off excess water with filter paper. Flood the preparation with fuchsin or with methylene blue. Allow the stain to act for about 2 minutes at room temperature. Wash thoroughly with water, drain, and allow the preparation to dry. Mount in Canada balsam.

Reference:

SHUNK, I. V. A modification of Loeffler's flagella stain. *Jour Bacteriol.*, 1920, 5, p. 181.

III. *Zettnow's method.*—Dissolve 10 grams tannin in 200 c.c. water, warmed to 50° or 60° C., and add 30 c.c. of a 5 per cent aqueous solution of tartar emetic. The turbidity of the mordant should entirely clear up on heating. The mordant should keep for months when a small crystal of thymol is added to it.

Next dissolve 1 gram silver sulphate in 250 c.c. distilled water. Of this solution take 50 c.c. and add to it drop by drop ethylamine (this comes in a 33 per cent solution) until the yellowish-brown precipitate which forms at first is entirely dissolved and the fluid is clear. Only a few drops are required. The bacterial preparations prepared as described above are floated in a little mordant contained in a Petri dish and heated over a water bath for 5 to 7 minutes. Take the dish containing the preparation off the water bath and, as soon as the mordant becomes slightly opalescent as the result of cooling, remove the cover glass preparation and wash thoroughly in water. Then heat a few drops of the ethylamine-silver solution upon the mordanted cover preparation until it just steams and the margin appears black. Next wash thoroughly in water and mount. This method gives very satisfactory results.

Reference:

Zettnow's Method. STITT. *Practical Bacteriology* (ed. 7; Blakiston's Sons, Philadelphia, 1923), p. 63.

Giemsa's stain.—

The formula is as follows:

Azur II—Eosin	3 gms.
Azur II	0.8 gm.
Glycerin (Merck, chemically pure)	250 gms.
Methyl-alcohol (Kahlbaum I)	250 gms.

The staining fluid is manufactured by Grübler, and it is best to obtain it already prepared. As Giemsa has modified the formula for another purpose, it is desirable to specify 'old' Giemsa stain when ordering.

(1) The preparation is dried in the air and fixed in alsolute alcohol 15 minutes, or in methyl-alcohol 2 or 3 minutes, after which the alcohol is removed with filter paper.

(2) To 1 c.c. of distilled water in a small graduate add 1 drop of the staining fluid and shake gently. This dilution is to be made immediately before proceeding to the next step.

(3) Cover the preparation with freshly diluted staining fluid for 10 to 15 minutes. For the demonstration of spirochaetes stain for from 1 to 2 hours, or longer.

(4) Wash in a stream of water.

(5) Remove excess of water with filter paper, dry in the air, and mount in balsam.

If especially intense staining is desired, add to the distilled water, before mixing it with the stain, a little potassium carbonate solution in the proportion of 1 or 2 drops of a 1 per cent solution to 10 c.c. of water.

Use: For blood smears and for the demonstration of spirochaetes.

If the dilution of the dye is made with 1:1000 potassium or sodium carbonate solution it stains more deeply.

Reference:

MALLORY AND WRIGHT. (Giemsa's Stain) (ed. 4; Philadelphia, W. B. Saunders Co., 1908, p. 425.

Gram stain.—

I. *Gram's method.—*

(1) Make thin films and fix by heat.

(2) Apply one of the following stains for 1½ minutes:

Ammonium oxalate—gentian violet (undiluted)
Carbol gentian violet (undiluted)
Carbol methyl violet (undiluted)
Anilin water gentian violet (undiluted)

If Sterling's gentian violet is used apply concentrated stain for 30 seconds.

(3) Wash in tap water.

(4) Apply Lugol's iodine solution for ½–1 minute, until the smear turns dark brown.

(5) Decolorize with acetone-alcohol until the violet color ceases to wash away.

(6) Wash in tap water.

(7) Counterstain with either one of the following stains:

Carbol fuchsin (diluted 1:10) for 1 minute.
Safranin, 3 per cent aqueous solution for 2 minutes.
Bismark brown, 0.2 per cent aqueous solution for 2 minutes.

II. *Modified Gram stain—(Hucker and Conn).—*

(1) Air dry a thinly spread film and fix by heat.

(2) Stain with ammonium oxalate gentian violet solution (p. 380) for 1 minute (better results are sometimes secured by flooding the slide with the stain and washing immediately with water).

(3) Wash in water.

(4) Apply Lugol's solution for 1 minute.

(5) Wash in water.

(6) Decolorize in 95 per cent or absolute alcohol for 30 seconds with gentle agitation.

(7) Counterstain with safranin (1:10 prepared from the saturated solution).

Reference:

HUCKER, G. J., AND CONN, H. J. Methods of Gram staining, N. Y. Agr. Exper. Sta. *Tech. Bull.*, 1923, p. 93.

III. *Modified Gram stain.—(Kopeloff and Beerman's).—*

(1) Air dry a thinly spread film and fix with the least amount of heat necessary to kill the organisms.

(2) Flood slide with dye solution. This is prepared by previously mixing in a beaker about 30 drops of a 1 per cent aqueous solution of methyl violet 6B (Coleman and Bell) with 8 drops of a 5 per cent solution of sodium bicarbonate. Allow the mixture to remain on the slide 5 minutes or more.

(3) Flush off the excess stain with the iodine solution and cover with fresh iodine solution for 2 minutes or longer. The iodine solution consists of 2 gm. iodine dissolved in 10 c.c. normal sodium hydroxide solution, to which is then added 90 c.c. of water.

(4) Drain off the excess iodine solution, without blotting (no water being used), but the film is not permitted to become dry.

(5) Add acetone (100 per cent) drop by drop until no color is seen in the drippings from the slide, which is slighly tilted. This usually requires less than 10 seconds and should be reduced to a minimum.

(6) Air dry the slide.

(7) Counterstain for 10–30 seconds with 0.1 per cent aqueous solution of basic fuchsin.

(8) Wash off excess stain by short exposure to tap water and air dry. If slide is not clear immersion in xylol has been recommended.

Reference:

KOPELOFF AND BEERMAN. Modified Gram Stains. *Jour. Infect. Dis.*, 1922, 31, p. 480.

Greenthal's Kresylecht violet methylene blue stain for C. diphtheriae.

Kresylecht violet	0.07 gms.
Methylene blue	0.1 gms.
Glacial acetic acid	3.00 gms.
Water	100.00 gms.

Dissolve the dye in the acetic acid and water solution and filter.

(1) To a drop of water on a cover glass make a smear of the growth with a platinum wire.

(2) Dry gently over flame.

(3) Place one drop of the stain on the cover glass. Allow it to remain for from 1 to 2 minutes and then shake off the excess stain. Place a drop of water on the cover and make a hanging mount with a hollow ground slide. The preparation can be sealed with the excess water. The polar granules are purplish red and the cell body is light greenish blue. Most of the other organisms stain very lightly, excepting a few strains of cocci. The stain can be used with the usual slide method. The polar granules by this method are purplish red and the cell body is light blue.

Spore Stain

I. *Burke's stain.*—

(1) Prepare smear.

(2) Immerse slides for 2 minutes in a beaker of steaming carbol fuchsin.

(3) Wash with distilled water immediately after removal from the stain and blot dry.

(4) Decolorize with absolute acetone, the slide being flooded 2 or 3 times and the acetone poured off almost at once.

(5) Wash with water and blot dry.

(6) Counterstain with dilute Loeffler's methylene blue for 2 minutes.

Reference:

Burke. Differetial staining of living and dead spores. *Jour. Infect. Dis.*, 1923, 32, p. 434.

II. *Moeller's stain.*—Cover slips are prepared as usual and fixed in the flame.

(1) Wash in chloroform for 2 minutes.

(2) Wash in water.

(3) Cover with 5 per cent chromic acid ½ to 2 minutes.

(4) Wash in water. Invert and float cover slips on carbol-fuchsin solution in a small porcelain dish. Heat gently with a flame until it steams; continue this for 3 to 5 minutes. (This step can also be done by covering the cover glass with carbol fuchsin and holding over the flame.)

(5) Decolorize with 5 per cent sulphuric acid 5 to 10 seconds.

(6) Wash in water.

(7) Stain with aqueous methylene blue ½ to 1 minute. By this method spores will be stained red, the body blue.

Reference:

Moeller's Stain: Hiss-Zinsser. *A Textbook of Bacteriology* (ed. 5), p. 116.

*Tilden's spirochaeta stain.**—A buffer solution containing formaldehyde (1 part of formaldehyde losution in 9 parts of phosphate buffer solution) is used as a fixative. The scraping or tissue emulsion is suspended in a small amount of the buffered formaldehyde solution and the mixture allowed to stand for at least 5 minutes (the longer the fixation the better the results; the organisms remain well preserved for at least 2 or 3 weeks). These films are made on clean slides, dried in the air, the film surface is flooded with a saturated alcoholic solution of gentian violet such as is used for Gram's stain, and the slide is almost immediately washed in running water and air dried. If the amoun of material available is very small, a drop of the fixative (from a capillary pipette) may be put on a slide and drop of the exudate added. The mixture is allowed to stand for 5 minutes or longer (protected from evaporation) and then spread into a very thin film. The less blood serum present, the better the differentiation between the organisms and the background. If Giemsa solution is used for staining, the slides are left in the staining fluid for one hour, as usual. It may be mentioned that the organisms remain well preserved for at least 2 or 3 weeks in tissues fixed in buffered formaldehyde solution and may be teased out for dark-field examination or for staining by the procedure outlined above. They take a more intensive stain when teased out of tissues.

Reference:

Noguchi, H., A note on the venereal spirochetosis of rabbits. *Jour. Am. Med. Assoc.*, 1921, 77, p. 2052.

Wayson's stain.—For plague bacilli.

Methylene blue	1.5 gms.
Acid carbolic (99.5 per cent)	5.0 c. c.
Distilled water	100.0 c. c.
Absolute alcohol	10.0 c. c.

To 5 c.c. of the foregoing solution add 0.15 to 0.25 c.c. saturated alcoholic fuchsin and 5 c.c. of 5 per cent carbolic acid. Filter. The solution will not overstain and acts in a few seconds.

Reference:

Personal communication.

Wright's stain.—

Sodium bicarbonate	0.5 gm.
Water	100.0 c. c.
Methylene blue B. X. or medicinally pure	1 gm.

* Eighty-eight parts of M/15 Na_2HPO_4 + 12 parts of M/15 KH_2PO_4 (Ph 7.6). Nine parts of this solution are added to 1 part of commercial formaldehyde solution. The mixture keeps well for from 2 to 3 weeks at room temperature.

The mixture should be in a flask of such size that it forms a layer not more than 6 cm. deep.

(1) After heating allow the mixture to cool. Filter to remove the precipitate which has formed. The color of the cold solution when viewed in a thin layer by transmitted yellowish artificial light should be a deep purple red.

(2) To each 100 c.c. of the filtered mixture add 500 c.c. of a 1 per cent aqueous solution of yellowish water soluble eosin and mix thoroughly. Collect the abundant precipitate which immediately appears on a filter.

(3) When the precipitate is dry dissolve it in methyl alcohol (Merck's) using 0.1 gm. precipitate to 60 c.c. alcohol. To facilitate solution, the precipitate is to be rubbed up with the alcohol in a porcelain dish or mortar with a spatula or pestle. Heat the mixture in a steam sterilizer at 100° C. for 1 hour counting the time after the sterilizer has become thoroughly heated.

Use: As a blood-staining fluid.

Mode of application:

(1) Cover the film with a noted quantity of the staining fluid by means of a medicine dropper or Pasteur pipette.

(2) After one minute add to the staining fluid the same quantity of distilled water and allow the mixture to remain for 2 or 3 minutes, according to the intensity desired. A delicate scum with an iridescent metallic luster forms on the surface. A longer period of staining may produce a precipitate. Eosinophilic granules are best brought out by a short period of staining. The quantity of the diluted fluid on the preparation should not be so large that some of it runs off.

(3) Wash the preparation in water for 30 seconds or until the thinner portions of the film become yellow or pink in color.

(4) Dry and mount.

Fresh films should be used. The water used for dilution and washing should have a neutral or a slightly alkaline reaction. If the yellow color in water in a test tube to which has been added a small pinch of haematoxilin does not change to a violet color in a few minutes it should be treated with 1 per cent sodium carbonate until it does so.

Reference:

WRIGHT, J. H. Directions for making and using the Wright blood-stain. *Jour. Am. Med. Assoc.*, 1910, 55, p. 1979.

BIOCHEMICAL TESTS

Indol test.—Culture grown in media 19.

(*a*) *Ring test.*—To 5 c.c. of culture add 1 c.c. of 10 per cent H_2SO_4, shake, and let stand 20 minutes. A red color shows the presence of nitrites and indol. This reaction is known as the 'cholera red' reaction. If no red color appears, let 3 to 4 c.c. of a 1:1000 $NaNO_2$ run carefully down the side of the tube so as to form a sharp line of contact. Allow the layered culture to stand for half an hour and look for a pink color at the line of juncture which indicates the formation of indol. When $NaNO_2$ has to be added, in addition to the H_2SO_4, the reaction is known as the Salkowski test.

It is important to know that the peptone used will give the reaction. Certain preparations do not give the test when authentic strains of *V. cholera* are grown therein.

(*b*) *Ehrlich's test.*—

Solution I.

Paradimethyl-amido-benzaldehyde	4 gms.
96 per cent alcohol	380 c. c.
Concentrated HCl	80 c. c.

Solution 2.

Saturated aqueous solution of potassium persulfate ($K_2S_2O_8$) prepared by saturating 100 c. c. *cold* distilled water with 1.8–2 grams of the salt (the salt decomposes when heated).

Add 2 c.c. of reagent No. 1 (*Paradimethyl-amido-benzaldehyde*) to 5 c.c. culture, then add 2 c.c. of reagent No. 2 and shake. Red color which becomes darker on standing and is soluble in amyl alcohol or chloroform indicates indol.

(*c*) *Vanillin test.*—To 5 c.c. of culture add 5 drops of a 5 per cent vanillin solution in 95 per cent alcohol, and 1 c.c. of concentrated hydrochloric acid or sulphuric acid. If indol is present, an orange color develops within 2 or 3 minutes. Tryptophane gives a reddish violet color which grows deeper on standing.

(*d*) *Gnezda oxalic acid reaction.*—Dip a strip of filter paper or a sterile cotton plug in saturated oxalic acid solution and allow to dry. Place the paper inside over the plug or insert the treated cotton plug in the culture tube to be tested for indol. Warm the tube to 37° C. or incubate for 24 hours. A pink color on the paper or plug indicates the presence of indol. Why?

Reference:

Holman and Gonzales. *Jour. Bacteriol.*, 1923, 8, p. 577.

(*e*) *Goré's test.*—Remove the plug of the culture (which must be of white absorbent cotton). Moisten it first with 4 to 6 drops of reagent No. 2 and then with 4 to 6 drops of solution No. 1 (as used in Ehrlich's test). Replace the plug and push it down until it is an inch or an inch and a half above the surface of the culture. Place the tube upright in a boiling water bath and heat for 15 minutes without letting the culture solution come in contact with the plug. The appearance of a red color indicates the presence of indol.

Reference:

GORÉ, S. N. *Indian Jour. Med. Res.*, 1921, 8, p. 505.

Test for the Reduction of Nitrates to Nitrites.—Organism grown in medium No. 50, 51, or 52.

Solution 1.

Sulphanilic acid	0.5 gms.
Acetic acid, Sp–gf. 1.04	150 c. c.

Acetic acid of Sp-gr. 1.04 is prepared by diluting 400 c.c. of concentrated acid (sp. gr. 1.75) with 700 c.c. of water.

Solution 2.

A-naphthylamine	0.1 gms.
Water	20 c. c.

Boil and, if necessary, filter. To clear filtrate, add 180 c.c. of acetic acid sp. gr. 1.04.

Mix in a test tube 2 c.c. each of reagents 1 and 2 and add 4 c.c. of culture to be tested. Place tube in warm water for 20 minutes. A red color indicates the presence of nitrites.

Reference:

HISS AND ZINSSER. *A Textbook of Bacteriology* (ed. 5; New York, D. Appelton & Co., 1924), p. 200.

Test for the Production of NH_3.—Organism grown in medium No. 41, 52, or 58.

Add to 5 c.c. of the culture 0.5 c.c. of phenol red. If the P_H is above 8.0, moisten a piece of red litmus with neutral distilled water and fasten it between the cotton plug and the wall of the tube. Warm the tube in a water bath. Record the color of the litmus paper (a blue color indicates the presence of NH_3) and note the odor.

Test for the production H_2S.—Organisms grown in media . . . . .

Dip a piece of filter paper in a 3 per cent solution of lead acetate. Place the moist paper over the inside of the cotton plug and warm the culture. A black, brownish color of the filter paper indicates the presence of hydrogen sulphide. Lead acetate agar (No. 37) demonstrates the production of H_2S.

Methyl red test.—

Inoculate 2 per cent glucose peptone solution medium No. 59 with the organism to be studied. Incubate for 3 days. To 5 c.c. of the culture add 5 drops of methyl red indicator. A distinct red color is recorded as methyl red positive, a distinct yellow color as methyl red negative.

Acetyl-Methyl carbinol test.—

Voges-Proskauer test.—For this test, fill fermentation tubes with a 2 per cent glucose peptone solution and sterilize, or use a part of the culture grown to demonstrate the production of acid (methyl red test). After inoculation with the organism to be tested, incubate for 3 days. Then add 2 to 3 c.c. of strong caustic potash solution. The development of a pink color on exposure to the air is a positive reaction, the color being that of a weak eosin solution.

Reference:

Voges-Proskauer Reaction. STITT (ed. 7; Philadelphia, Blakiston's Sons, 1923), p. 36; also LEVINE, On the Significance of the Voges-Proskauer Test. *Jour. Bacteriol.*, 1916, 1, p. 153.

APPENDIX B

PREPARATION OF CULTURE MEDIA

PREPARED BY

P. SCHOENHOLZ

APPENDIX B

PREPARATION OF CULTURE MEDIA

PREPARED BY

P. SCHOENHOLZ

---

1. Alkaline Egg Medium

The yolk of one egg and the white of two are beaten up in a breaker; add 6 c.c. of normal sodium hydrate solution; add gradually 500 c.c. of tap water. Heat slowly to 95° C., keeping the mixture at this temperature for an hour or longer; filter through cotton wool and muslin. Tube. Autoclave at 115° C. for 20 minutes. Incubate 48 hours.

Use: In the cultivation of anaerobes.

2. Aronson's Medium

Leibig's meat extract or peptic digest agar	100 c.c.

Add enough sterile 10 per cent Na_2CO_3 to bring the reaction to P_H 8.4–8.6. Add:

Saccharose (sterile 25 per cent solution)	4 c.c.
Dextrin (sterile 25 per cent solution)	4 c.c.
Saturated alcoholic solution basic fuchsin	0.4 c.c.
Fresh sterile sodium bisulfite	1–2 c.c.

Enough sodium bisulfite should be added to decolorize the media to a light pink (see Endo agar). Cool to 60° C.; pour plates; keep in a dark, cool place.

Use: For the isolation of *V. cholerae.*

3. Ascitic Fluid Agar

Veal agar (3 per cent) is melted by boiling in a steam kettle. Shake the flask thoroughly making sure that all of the agar is melted. Cool down to 50° C. Add aseptically 33 per cent sterile ascitic fluid. Mix well by shaking and tube or pour plates. Incubate 24 hours. Keep in the ice box.

4. Avery's Broth (Artificial Mouse)

Add 0.1 per cent sterile sheep or rabbit blood to from 6–7 c.c. glucose veal broth P_H 7.6. Incubate at 37° C. to test sterility.

Use: As a routine medium for the enrichment of more delicately growing organisms.

5. Beef Heart Medium

Beef heart defatted and ground	500 gms.
Tap water	1000 c.c.

Infuse in the ice box over night. Boil ½ hour. Drain juice from meat. Add 2 parts peptic digest to 1 part beef heart juice. Adjust reaction with 2N Na_2CO_3 to P_H 7.4–7.6. Boil 10 minutes. Check reaction. Tube or bottle with about 2 cm. depth of meat finely ground and 5–6 cm. depth of broth. Add 0.5 cm. layer of vaseline if desired. Autoclave for one hour at 15 pounds pressure. Incubate 12 to 14 hours. Autoclave one hour at 15 pounds pressure. Incubate 48 hours. Check reaction, final P_H 7.2–7.4.

Use: Preservation and cultivation of anaerobes.

6. Blood Agar

Melt 1 liter or a smaller amount of Liebig's Meat Extract, "Hormone" or veal agar P_H 7.4. Cool to a temperature of 45° C. Add aseptically 5 per cent sterile defibrinated sheep's blood. Shake carefully and pour into sterile Petri dishes. Flame the neck of the flask after pouring each plate. Incubate at 37° C. 24 hours.

7. Blood Agar (Cooked) Sometimes Called "Chocolate" Agar

Prepare in the same manner as blood agar. The reaction of the medium should be P_H 7.8. After the blood has been added raise the temperature of the water bath to from 70–75° C. Keep the medium at that temperature for from 10–15 minutes or until it has uniformally turned brown. Cool to 45° C. Pour plates and incubate at 37° C. for 24 hours.

Use: For the isolation and cultivation of *B. influenzae.*

8. Blood Agar (Laked)

To 5 c.c. of blood add 5 c.c. of sterile distilled water. Add the laked blood aseptically to cooled melted agar as in formula No. 6.

Use: In the cultivation of meningococci.

9. Blood Broth (Cooked) Sometimes Called "Chocolate" Broth

To veal infusion broth P_H 7.6–7.8 add 5 per cent sterile rabbit's or sheep's blood. Heat to 75° C. for 10–15 minutes. Filter aseptically through sterile filter paper or a loosely packed cotton filter. Heat in the Arnold at 90° C. for 20 minutes on 2 successive days. Incubate 48 hours to test sterility.

Use: For the cultivation of *B. influenzae.*

10. Bordet Gengou Medium

Water	1000 c.c.
Glycerin	80 c.c.
Potato	500 gm.

Heat in autoclave 15 lbs. pressure for ½ hour. Pour off liquid. To 500 c.c. of this potato extract add salt solution (0.6 per cent) 1500 c.c. and agar 60 gms. Autoclave to dissolve. Check P_H 6.6–6.8. Filter through cotton pads. Distribute 250 c.c. amounts in one litre flasks. Autoclave ½ hour. Cool to 45° C. Add aseptically 60 to 250 c.c. defibrinated rabbit blood. Tube and slant. Incubate 48 hours.

Use: For the growth of *B. pertussis.*

11. Brain Medium

Sheep brains are cleaned from all tissue and blood, macerated and measured. Add one-third of their volume of 2 per cent peptone solution. Boil 15 minutes in a double boiler. Beat thoroughly with an egg beater. Tube and autoclave for 1 hour at 15 pounds pressure. Note the natural reaction. Incubate 48 hours and test the sterility by transplanting several loopfuls of media to agar stabs and from 1 to 2 c.c. in a tube of glucose broth.

12. Brain Suspension

Beef brain is freed from all blood clot and tissue. Add one-third its volume of distilled water and bring to a boil. The mixture is then beaten with an egg beater until it resembles thick cream. Then squeeze through gauze. Tube in lots of 12–15 c.c. each and sterilize in the autoclave for 1 hour at 15 pounds pressure. Incubate for 48 hours and test the sterility by transplanting several loopfuls of media to agar stabs and from 1 to 2 c.c. in tubes of glucose broth.

13. Brilliant Green Eosin Agar (Modified Teague's)

Liebig's (Ph 7.0), or preferably Peptic Digest agar melted and cooled to 60° C.	200 c.c.
Sterile 25 per cent Lactose solution	8 c.c.
Sterile 25 per cent Saccharose solution	8 c.c.
Sterile 3 per cent water soluble Eosin (aqueous solution)	4 c.c.
1/6 of 1 per cent brilliant green	4 c.c.

(Dilute 1 c.c. of 1 per cent brilliant green in 50 per cent alcohol in 5 c.c. distilled water.)

Pour aseptically into clay top Petri dishes. Note: The amount of brilliant green used will vary with the reaction of the medium. For each lot of media prepared 1/5 per cent and 1/6 per cent concentrations should be tested.

Use: Plating feces and duodenal contents.

14. Brom Cresol Purple Milk

Remove the cream from fresh milk. The fat-free portion is poured off into a sterile graduate cylinder, care being taken to avoid pouring any of the sediment which may have settled at the bottom of the tube. To one litre of milk add 40 c.c. of a 0.04 per cent aqueous solution of brom cresol purple. Note: Prepare this solution by dissolving 0.4 gram of brom cresol purple (Hynson Westcott Dunning, Baltimore) in a minimum amount of alcohol. Make up to one litre with distilled water.

Tube and sterilize in the Arnold at 100° C., 30 minutes on the first day, 15 minutes on the 2 following days. Incubate at 37° C. for 48 hours to test sterility.

Acidity	Indicator, reaction, etc.	Approximate Ph value
"Neutral"	Same color with brom cresol purple as sterile milk—i. e., blue to gray-green	6.2–6.8
"Weak"	Color with brom cresol purple lighter than in sterile milk—i. e., gray-green to greenish yellow	5.2–6.0
"Moderate"	Yellow with brom cresol purple. Not curdled	4.7–6.0

Reference:

Clark, W. M., and Lubs, H. A. A substitute for litmus for use in milk cultures. *Jour. Agr. Res.*, 1917, 10, pp. 105–111.

15. Carbohydrate Medium

Distilled water	1000 c.c.
Peptone (Difco)	10 gms.
Dibasic potassium phosphate	2 gms.

Heat until dissolved. Make up to volume. Adjust reaction P_H 7.0. Autoclave for 15 minutes at 15 lbs. pressure to precipitate the phosphates. Filter through paper. Flask in 200 c.c. amounts and autoclave for 30 minutes at 15 pounds pressure. Add to each flask sugar desired (1 per cent and Andrade's indicator (1 per cent). Tube in Durhams tubes (test tubes with inverted glass capsules), sterilize 12 minutes at 15 pounds in the autoclave. Incubate 48 hours.

Use: For fermentation reactions, particularly in the identification of gram negative rods.

16. Hiss Serum Water

Tap water	3 parts
Peptone (Witte)	0.5 per cent
Beef or sheep serum	1 part

Add the peptone to the water and adjust the reaction to P_H 7.2. Add the serum to the peptone solution. Distribute in flasks in measured amounts. Heat in Arnold for 30 minutes at 100° C. on 3 consecutive days. Add 1 per cent of the desired sterile sugar solutions* to each part of serum and 1 per cent Andrade's Indicator. Tube aseptically in small test tubes and sterilize in the Arnold sterilizer for 15 minutes.

*Note.—In preparing inulin Hiss serum water dissolve the inulin in distilled water and sterilize in the autoclave for 15 minutes at 15 pounds pressure. The serum is then diluted with this sterile inulin solution, tubed, and sterilized as usual.

Use: For fermentation reactions, particularly suitable for more delicately growing organisms.

17. Dorsett's Egg Medium

Partially sterilize the outer shells of the eggs by wrapping them in cotton soaked with 70 per cent alcohol. Let the alcohol act for 20 minutes. Carefully break eggs, and drop the contents into a wide-mouthed flasks. Break up the yoke with a sterile glass rod and shake the flask until the whites and the yokes are thoroughly mixed. Add 25 c.c. of distilled water to every four eggs; strain through sterile

cloth. Pour 10 c.c. into sterile test tubes and slant in an inspissator. Expose to 73° C. for 4 to 5 hours on 2 consecutive days. On the third day raise the temperature to 76° C. Sterilization may be finished by a single exposure to 100° C. in the Arnold sterilizer for 15 minutes. Incubate at 37° C. for 48 hours. Store in the ice chest. Before inoculation add 2 or 3 drops of sterile water to each tube.

Use: For the cultivation of the tubercle bacillus.

18. Dieudonne's Medium

Mix 15 c.c. defibrinated sheep's blood with 15 c.c. in KOH. Sterilize for 30 minutes at 100° C. in the Arnold. (This keeps several months.) Add the mixture to 70 c.c. of 3 per cent neutral Liebig's extract or veal infusion agar. Pour into Petri dishes, remove covers, invert the dishes and dry in the incubator. The medium should not be used for 24 hours since the ammonia given off would inhibit the growth of the organisms.

Use: In the isolation of *V. cholerae.* Inhibits the growth of other intestinal organisms.

19. Dunham's Peptone Solution

Distilled water	1000 c.c.
Witte's Peptone	10 gms.
Sodium Chloride	5 gms.

Heat until the ingredients are thoroughly dissolved and correct the reaction to P_H 7.0. Autoclave at 15 pounds pressure to precipitate phosphates. Make up to volume. Filter until perfectly clear. Tube and sterilize in the autoclave at 15 pounds pressure for 30 minutes.

Use: To demonstrate the production of indol.

20. Endo Agar

Liebig's Extract or Peptic Digest Agar	200 c.c.
Lactose (dry)	2 gms.
Or	
25 per cent sterile solution	8 c.c.
Sterile 10 per cent sodium carbonate to bring the reaction to Ph 7.8	1–2 c.c.

Place in the Arnold sterilizer at 100° C. for 15–20 minutes. Cool to 60° C. and add

Saturated alcoholic solution of basic fuchsin	4 c.c.
Sodium bisulfite, 10 per cent	10 c.c.

or enough to decolorize the fuchsin to a light rose-pink color.

Pour aseptically into sterile Petri dishes. The medium when cool has a very pale pink or salmon color. It should be kept in the dark as exposure to light will turn the medium red.

Use: For plating feces and in water analysis.

21. Eosin Methylene Blue Agar

Melt one flask (1000 c.c.) of peptic digest agar (2 per cent). Check P_H 6.8–7.2. Add 10 grams of dry lactose. Boil until dissolved. Or, add 20 c.c. of a 50 per cent sterile solution of lactose. Cool to 50–60° C., then add

15 c.c. of 2 per cent eosin (water soluble).
20 c.c. of 0.5 per cent aqueous methylene blue.

Mix by rotating the flask. Pour aseptically into clay or glass top Petri dishes.

Note.—The solutions can be kept sterile in small containers. Sterilize by heating at 15 pounds pressure for 12 minutes.

22. Gelatin
(high melting point)

Sterile single strength veal or peptic digest broth heated to 60° C.	1000 c.c.
Gelatin (gold label)	200 gms.

Put in double boiler to dissolve. Neutralize the reaction with 2N NaOH so that the medium has a reaction of about P_H 7.4. Add the beaten white of one egg. Heat the water in the outer boiler to boiling. Stir constantly. Close the pot containing gelatin with a cover and boil 15 minutes or until all the egg has coagulated. Filter into a sterile flask through sterile gauze and cotton previously wetted with boiling water. Tube in 120 x 13 m.m. tubes with aseptic precautions and sterilize for 20 minutes in the Arnold at 100° C. Place tubes in ice box. Repeat the sterilization on the second and third days and cool as before. Test sterility and then store in the ice chest.

23. Glucose Beef Juice Broth

Lean ground beef	500 gms.
Distilled water	1000 c.c.

Allow to infuse over-night in the ice box. Squeeze out the juice of the meat through a double layer of fine mesh gauze. Bring to a boil slowly, stirring constantly. Filter through a double layer of fine mesh gauze. Add

Peptone (Witte)	15 gms.
Sodium chloride	5 gms.

Dissolve ingredients and adjust the reaction to P_H 7.8. Make up to volume. Autoclave at 15 pounds pressure for 30 minutes. Allow the precipitate to settle. Syphon aseptically. Add 1 per cent glucose in the form of a sterile 50 per cent solution. Tube aseptically in Smith fermentation tubes or in large test tubes. Heat in the Arnold at 100° C. for 15 minutes. Incubate at 37° C. for 48 hours.

Use: For testing the sterility of bacterial products (in Smith fermentation tubes); for growing streptococci.

24. Glucose Brain Broth

Distilled water	1000 c.c.
Liebig's extract	3 gms.
Peptone "Difco"	10 gms.
Sodium chloride	8 gms.
Dibasic potassium phosphate	2 gms.
Glucose	2 gms.

Dissolve ingredients. Adjust the reaction P_H 7.8. Add 20 c.c. of Andrade's indicator. Place a layer of brain (1–2 cm. in depth) in nursing bottles or in tall tubes. Add 100 c.c. of broth to each bottle or 15 c.c. to each tube. Autoclave for 20 minutes at 15 pounds pressure.

Use: For the cultivation of micro-aerophilic organisms.

25. Glucose Peptic Beef Heart Broth

Beef heart	500 gms.
Distilled water	1000 c.c.

Infuse over-night in the ice box. Boil ½ hour. Drain juice from meat. Add two parts peptic digest broth to one part beef heart broth. Adjust reaction P_H 7.8–8.2. Add 0.5 per cent glucose. Tube 10 c.c. per tube with three or four small pieces of meat in each tube. Stratify with a 0.5 cm. layer of vaseline. Run in open steam in the autoclave for ½ hour, then raise the pressure slowly to 15 pounds and autoclave 1 hour. Incubate 12–14 hours. Resterilize at 15 pounds pressure for 1 hour. Incubate 24 to 48 hours. Check reaction P_H 7.2–7.4.

Use: To check positive and negative cultures by gas production in determining thermal death point.

26. Glucose Peptic Beef Heart Blood Agar

Peptic digest	2 parts
Beef heart broth	1 part
Agar shreds	2 per cent

Boil in steam kettle until all of the agar is dissolved. Make up to volume. Adjust the reaction P_H 7.4. Cool to 55° C. Add the white of 1 egg per liter. Stabilize in 1 litre flasks in autoclave for ½ hour at 15 pounds pressure. Filter through cotton pads just wetted with boiling water. Fill flasks or nursing bottles with 200 c.c. amounts. Autoclave ½ hour at 15 pounds pressure. Incubate at 37° C. for 24–48 hours. When ready for use melt agar. Add 0.5 glucose in the form of a 50 per cent sterile solution. Cool to 45° C. Add 5 per cent sterile sheep or horse blood. Mix carefully and pour plate.

Use: For anaerobic plate counts.

27. Glucose Peptic Beef Heart Broth

Peptic digest	2 parts
Beef heart broth	1 part

Boil and readjust P_H 7.6 with 2N Na_2CO_3. Stabilize ½ hour in autoclave at 15 pounds pressure. Filter through paper. Add 0.5 per cent glucose. Fill into 9 c.c. amounts in test tubes and autoclave for 30 minutes at 15 pounds pressure. Check reaction P_H 7.0–7.2. Incubate 48 hours.

Use: For estimating the number of spores in anaerobic cultures by the dilution method.

28. Glucose Peptone Solution (Standard)*

Peptone medium for the methyl red test.—

To 800 c.c. of distilled water add 5 grams Proteose-Peptone, Difco, or Witte's Peptone (other peptones should not be substituted); 5 grams c. p. dextrose; and 5 grams dipotassium hydrogen phosphate (K_2HPO_4). A dilute solution of the K_2HPO_4 should give a distinct pink with phenolphthalein. Heat with occasional stirring over steam for 20 minutes. Filter through folded filter paper, cool to 20° C. and dilute to 1000 c.c. with distilled water. Distribute 10 c.c. portions in sterilized test tubes. Sterilize by the intermittent method for 20 minutes on three successive days.

Use: To test for the production of acid (methyl red test) and for the production of acetyl-methyl carbinol (Voges-Proskauer reaction).

* All formulae marked "Standard" are those recommended in the *Manual of Methods for Pure Culture Study of Bacteria* prepared by the Committee on Bacteriological Technique of the Society of American Bacteriologists. The mode of preparation is given in detail in *Standard Methods for the Examination of Water and Sewage* (ed. 5; Am. Pub. Health Assoc., New York, 1923).

29. Glucose Veal Broth for Blood Cultures

Double strength veal infusion	1000 c.c.
Tap water	1000 c.c.
Peptone Difco 1½ per cent	30 gms.
NaCl 0.5 per cent	10 gms.
Dibasic potassium phosphate 0.2 per cent	4 gms.

Boil until ingredients are dissolved. Adjust the reaction to the desired degree of alkalinity P_H 7.6–8.0. Stabilize in autoclave at 15 pounds pressure for ½ hour. Check P_H 7.6–8.0 and make up to volume with tap water. Filter through paper. Pour into nursing bottle, 100 c.c. per bottle. Autoclave ½ hour at 15 pounds pressure. Add aseptically to each bottle 1 per cent glucose (2 c.c. of a sterile 50 per cent solution). Arnold 20 minutes at 100° C. Incubate 48 hours.

30. Glycerin Peptic Digest Agar

To 2 per cent peptic digest agar (P_H 7.2) add 5 per cent C. P. glycerin. The mixture should be shaken well. Tube in 10 c.c. quantities and autoclave at 15 pounds pressure for 15 minutes.

31. Glycerin Peptic Digest Broth

To 1 liter of sterile peptic digest broth P_H 7.0 is added 5 per cent glycerin. The mixture is shaken well, tubed, and autoclaved for 15 minutes at 15 pounds pressure. Incubate 48 hours.

32. Glycerin Veal Agar

To 2 per cent melted veal agar (P_H 7.2) add 5 per cent C. P. glycerin. Mix thoroughly. Tube in 10 c.c. quantities and autoclave at 15 pounds pressure for 15 minutes.

33. Glycerin Veal Infusion Broth

Five per cent glycerin is added to stock veal infusion broth (P_H 7.2) and sterilized in the Arnold for 3 consecutive days or in the autoclave for 15 minutes at 15 pounds pressure.

Use: In the cultivation of the tubercle bacillus.

34. Greenspoon's Medium

Preparation.—To 75 c.c. of clear pig, sheep or human serum is added 1 c.c. of a 50 per cent sodium citrate solution and sufficient dextrose veal infusion broth to bring the volume to 100 c.c. Beef extract broth may be substituted for the meat infusion. With 3 per cent citric acid solution the reaction is then adjusted to P_H 6.4, using di-brom-thymol-blue as an indicator. Next the medium is delivered into culture tubes, coagulated in an inspissator and sterilized by the fractional method on three successive days.

Use: For the demonstration of *C. diphtheriae* from throat swabs, etc.

35. Lactose Beef Infusion Broth

Ground lean beef	500 gms.
Tap water	1000 c.c.

Infuse for 1–2 hours, then bring to a boil and strain through a double layer of gauze. Cool and defat by filtration through cotton filter pads wetted with cold water. Distribute in nursing bottles. Autoclave for 30 minutes at 15 pounds pressure. Add 1 per cent lactose in the form of a sterile 25 per cent lactose solution. Heat in the Arnold sterilizer at 100° C. for 20 minutes.

Use: For the preparation of acidophilus broth.

36. Lactose Peptone Bile

Whole filtered ox-bile	1000 c.c.
Lactose (C. P.)	10 gms.
Peptone (Difco)	10 gms.

Tube in small size Smith fermentation tubes and sterilize for 15 minutes at 15 pounds pressure, or put in flasks if desired.

Use: For the isolation of *B. typhosus* and for stool analysis.

37. Lead Acetate Agar

Liebig's extract or peptic digest agar	100 c.c.
Glucose (sterile, 25 per cent solution)	4 c.c.
Lactose (sterile, 25 per cent solution)	4 c.c.
Lead acetate (sterile, 0.5 per cent solution)	1 c.c.

Add the sterile lead acetate solution to the melted agar (containing the sugar solutions) and tube aseptically or, add the sterile lead acetate to the agar tubed in measured amounts. Do no slant.

Use: For the differentiation of organism belong to the Paratyphoid group.

38. Liebig's Agar for Milk Counts

MIXTURE A

Beef extract (Liebig's)	0.3 per cent total quantity
Witte's peptone	0.5 per cent total quantity
Distilled water	40.0 per cent total quantity

Place in kettle. Weigh kettle with contents. Heat on stove to boiling and boil 5 minutes. Adjust reaction to P_H 7.4. Boil five minutes. Bring back to volume with hot distilled water. Filter through paper.

MIXTURE B

Agar 1.5 per cent of total quantity. Soak and wash under tap in sieve. Weigh before and after soaking. Distilled water 60 per cent of total quantity of medium to be made, minus that absorbed by the agar during washing.

MXTURE A–B

(Agar not yet melted)

Heat over stove, stirring at frequent intervals until agar is entirely melted. Then boil and stir constantly for 20 minutes. Make up by weight water lost by evaporation by adding hot distilled water. Filter through filter paper in a Buchner funnel under vacuum until perfectly clear. Tube 12 c.c. in each tube. Cap with paper caps. Autoclave 20 minutes at 15 pounds. Incubate 24 hours.

Reference:

Standard Methods of Milk Analysis (ed. 4; Am. Pub. Health Assoc., 1923).

39. Liebig's Meat Extract Agar

Water	1000 c.c.
Liebig's Meat Extract	10 gms.
Peptone (Difco)	10 gms.
Sodium chloride	5 gms.
Dibasic potassium phosphate	2 gms.
Agar shreds	20 gms.

Dissolve the ingredients. Adjust the reaction to P_H 7.0–7.4. Make up to volume and recheck the reaction. Cool to 56° C. Add the beaten white of one egg. Autoclave for 45 minutes at 15 pounds pressure. Filter through gauze and cotton pads which have been moistened with boiling water. Check the reaction P_H 7.0–7.4. Fill into flasks in measured amounts. Sterilize ½ hour, 15 pounds pressure.

Use: For blood plates.

40. Liebig's Meat Extract Agar (Standard)

Add 3 grams of beef extract, 5 grams of peptone, and 12 grams of agar, dried for ½ hour at 105° C. before weighting, to 1000 c.c. of distilled water. Boil over a water bath until all agar is dissolved, and then make up the loss by evaporation. Cool to 45° C. in a cold water bath, then warm to 65° C. in the same bath, without stirring. Make up lost weight and adjust the reaction to a faint pink with phenol red, or to the required tint with brom thymol blue.

Use: Standard agar used in the examination of water and sewage.

41. Liebig's Meat Extract Broth

Water	1000 c.c.
Liebig's Meat Extract	10 gms.
Peptone (Difco)	10 gms.
Soduim chloride	5 gms.

Dissolve the ingredients, then make up to volume. Adjust the reaction to P_H 7.0–7.2. Heat in autoclave for 30 minutes, correct volume again and check the reaction. If necessary make corrections and boil again for 5 minutes. Filter solution through a double layer of filter paper; tube or flask. Sterilize in the autoclave for 30 minutes at 15 pounds pressure and incubate, or in the Arnold by the intermittent method.

42. Liebig's Nutrient Broth (Standard)

Add 3 grams of beer extract and 5 grams of peptone to 1000 c.c. of distilled water. Heat slowly on a steam bath to at least 65° C. Make up lost weight and adjust the reaction to a faint pink with phenol red, or to the required tint with brom thymol blue. Cool to 25° C. and filter through filter paper until clear. Distribute in test-tubes, 10 c.c. to each tube. Sterilize in the autoclave at 15 pounds (120° C.) for 15 minutes after the pressure reaches 15 pounds.

43. Liebig's Meat Extract Carbohydrate Broth (Standard)

Sugar broths shall be prepared in the same general manner as nutrient broth with the addition of 1.0 per cent of the required carbohydrate just before sterilization.

The removal of muscle sugar is unnecessary as the beef extract and peptone are free from any fermentable carbohydrates.

The reaction of sugar broths should be a faint pink with phenol red or the required tint with brom thymol blue.

Sterilization shall be in the autoclave at 15 pounds (120° C.) for 15 minutes after the pressure reaches 15 pounds, provided the total time of exposure to heat is not more than ½ hour; otherwise a 10 per cent solution of the required carbohydrate shall be made in distilled water and sterilized at 100° C. for 1½ hours, and this solution shall be added to sterile nutrient broth in amounts sufficient to make a 1.0 per cent solution of the carbohydrate and the mixture shall then be tubed and sterilized ăt 100° C. for 30 minutes, or it is permissible to add by means of a sterile pipette directly to a tube of sterile neutral broth enough of the carbohydrate to make the required 1.0 per cent.

The tubes so made shall be incubated at 37° C. for 24 hours as a test for sterility. (The choice of indicator is left to the individual worker.)

Use: Standard carbohydrate media used in the examination of water and sewage and recommended by the Committee on Bacteriological Technic of the Society of American Bacteriologists.

44. Liebig's Meat Extract Gelatin (Standard)

Add 3 gms. of beef extract and 5 gms. of peptone to 1000 c.c. of distilled water and add 100 gms. of gelatin dried for ½ hour at 105° C. before weighing.

Heat slowly on a steam bath to 65° C. until all gelatin is dissolved. Make up lost weight and adjust the reaction to a faint pink with phenol red, or to the required tint with brom thymol blue.

Filter through cloth and cotton until clear. Distribute in test tubes, 10 c.c. to each tube, or in larger containers as desired. Sterilize in the autoclave at 15 pounds (120° C.) for 15 minutes after the pressure reaches 15 pounds.

Note.—The solution of the gelatin will be facilitated by allowing it to soak in the cold ½ hour before heating.

45. Liver Infusion (Stock)

Ground beef liver	500 gms.
Tap water	1000 c.c.

Infuse in ice chest over night or for 3–4 hours at room temperature. Heat slowly to 100° C. Filter while hot through cotton. Sterilize in autoclave at 15 pounds pressure for 30 minutes.

46. Liver Infusion Agar for Anaerobes

Ground lambs or calves liver	500 gms.
Distilled water	2000 c.c.

Infuse in ice box overnight. Bring to a boil and strain through cheese cloth. Add

Peptone (Difco)	30 gms.
Sodium chloride	10 gms.
Agar shreds	30 gms.

Melt the ingredients and readjust the reaction to P_H 7.2–7.4. Bring up to volume. Cool to 50°–60° C. and add the beaten white of one egg per litre. Mix well. Pour into 1 litre flasks and stabilize in the autoclave for ½ hour at 15 pounds pressure. Filter through cotton pads, just moistened with boiling water, and check the reaction to P_H 7.2. Tube in 10 c.c. amounts and pack loosely in baskets. Autoclave for 30 minutes at 15 pounds pressure. Incubate 24 hours.

47. Liver Infusion Broth

Liver infusion	1000 c.c.
Difco Peptone	20 gms.
Sodium chloride	5 gms.

Boil to dissolve the ingredients. Adjust the reaction to P_H 7.4–7.6. Stabilize ½ hour at 15 pounds pressure. Filter through paper. Sterilize for 30 minutes at 15 pounds pressure. Incubate 24–48 hours.

48. Loeffler's Serum

Prepare 1 per cent dextrose veal broth and adjust reaction to P_H 7.2–7.4. Add 3 parts of beef serum.

If chloroform has been added to the beef serum as a preservative, place the mixture (or beef serum alone) in a flask in a water bath held at 45° C. under vacuum for 1 hour to expel the chloroform.

Place sufficient amount of the mixture in sterile test tubes (size 150 x 10 mm.) for slanting. Put these in a slanting position in the inspissator and heat until solidified. The temperature should be brought up very slowly. After solidification of the media, sterilize on the second and third days by placing the tubes in the Arnold sterilizer, the rise in temperature should be very gradual. Heat at 95–100° C. for 20 minutes. In case all the ingredients are sterile and the tubing has been carried out aseptically, sterilization is unnecessary. Incubate 48 hours to test sterility.

Use: For the detection and cultivation of the diphtheria bacillus.

49. Nicolle's Medium

Agar	14 gms.
Sea salt	6 gms.
Water	900 c.c.

Pure sodium chloride may be substituted for sea salt. Melt, tube, and autoclave.

Use: For the cultivation of protozoa.

50. Nitrate Agar

Add 0.1 per cent potassium nitrate to formula 40.

51. Nitrate Broth

Distilled water	1000.0 c.c.
Witte's peptone	10.0 gms.
Potassium nitrate	0.2 gms.

Boil to dissolve peptone, and make up to volume. Stabilize at 15 pounds pressure for 30 minutes. Filter through paper. Tube in 5 or 10 c.c. amounts. Autoclave at 15 pounds pressure for 15 minutes.

Use: For the demonstration of the reduction of nitrates to nitrites.

52. Nitrate Broth (Standard)

Add 0.1 per cent potassium nitrate to formula 42.

53. Ox-Gall Medium

Nine parts of ox-bile are mixed with 1 part of glycerin, 2 per cent peptone is added, and the medium is heated in the Arnold sterilizer or in a double boiler until the peptone goes into solution. Adjust reaction to P_H 7.2. After heating in the Arnold sterilizer for 30 minutes filter through paper and tube in 10 c.c. quantities and sterilize in the Arnold for 20 minutes on 3 consecutive days.

Use: For *B. typhosus* blood cultures.

54. Pancreatic Digest of Casein

Obtain the fresh pancreas of a pig, free it from fat as far as possible, and weigh. Mince fine and add three times its weight of distilled water and its own weight of fresh alcohol. Shake well in a large bottle and allow to stand for 3 days at room temperature, shaking the bottle occasionally. Strain through muslin.

The filtrate which comes through very slowly is measured and treated with 1 c.c. of concentrated hydrochloric acid per litre. This causes the appearance of a cloudy precipitate which settles in a few days and can be filtered off. The fluid keeps, without any additional antiseptic, for an indefinite period if stoppered. If desired at once the extract may be used before adding the HCl, the function of which is to retard the slow auto-destruction of the trypsin.

Digestion of the casein.—To 1 litre of tap water add 20 grams of anhydrous sodium carbonate and boil in a 2-litre flask or a large evaporating basin; when boiling transfer the vessel to a boiling water bath and gradually add 200 grams of granulated casein. This must be dusted in carefully and stirred well to avoid the formation of lumps. Transfer to a clean Winchester quart bottle and wash out the mixing vessel with a litre of cold tap water, which is also transferred to the bottle. If necessary allow the temperature to fall to 40° C. and add 50 c.c. of the pancreatic extract and 15 c.c. of the chloroform. Shake well. Stopper with a loose cork and incubate at a temperature of from 37° C. to 40° C., shaking the bottle vigorously each day to break up any lumps. After 5 days digestion add another 50 c.c. of the pancreatic extract and allow the mixture to stand for another 10 days; that is, not less than 15 days digestion in all.

Preparation of the stock broth.—The warm mixture is well shaken to break up any crusts of tyrosine that may have formed and is transferred to a large (3 litre) flask, treated with 400 c.c. of normal HCl (or 40 c.c. of pure concentrated HCl diluted with 360 c.c. of water), shaken, steamed for 30 minutes, and filtered. The filtrate is treated with 120 c.c. of normal sodium hydroxide and the reaction is adjusted.

Preparation of tryptic broth.—The stock broth is diluted with twice its volume of 0.5 per cent sodium chloride dissolved in tap water and the reaction is adjusted. The broth is distributed in containers, sterilized in the autoclave and incubated to test the sterility.

55. Pea Peptic Gelatin

Canned peas	500 gms.
Distilled water	1000 c.c.

Mash peas, boil ½ hour and strain through gauze. Mix:

Pea extract	1 part
Peptic digest	1 part
Gelatin	10 per cent

Dissolve by boiling in a double boiler. Readjust the reaction to P_H 7.6–7.8. Cool to 50–55° C. and add the white of one egg per litre. Stabilize by boiling in double boiler ½ hour or in the Arnold for

½ hour at 100° C. Filter through cotton. Check reaction to P_H 7.4–7.6. Fill containers with required amounts, add a layer (0.5–1 cm.) of sterile vaseline, and Arnold 3 consecutive days at 100° C. for one hour.

Use: For the production of spores with anaerobic organisms.

56. Peptic Digest
(sometimes called martin's broth)

Minced pig stomach	100 gms.
Minced pig liver	100 gms.

Place in a large bottle. Add 100 c.c. warm tap water 55° C. and 10 c.c. concentrated hydrochloric acid (c. p.). Mix thoroughly. Digest for 22–24 hours in a water bath at 55° C. Test for digestion by means of the biuret reaction; if not complete, continue digestion.

Biuret Reaction

Peptic digest	5 c.c.
N/2 NaOH	2 c.c.
5 per cent copper sulphate solution	3 drops

Digestion is complete if a rose color is obtained. If the purple color is obtained, digestion should be continued. When the digestion has reached such a stage that a rose color is obtained strain through cotton and gauze. Autoclave at 15 pounds for 30 minutes. Adjust the reaction to P_H 6.6 with 2N Na_2CO_3. Boil thoroughly for 10 minutes. Bottle while hot. Sterilize in the autoclave at 15 pounds pressure for 30 minutes.

Use: As a base for media used in the cultivation of organisms belonging to the colon-typhoid-dysentery group. It may also be used for the cultivation of aerobic gram + rods, for anaerobes, but *not* for the cultivation of *Diplococcus lanceolatus, Micrococcus intracellularis meningiditis, Micrococcus gonorrhoeae, streptococci,* hemoglobinophilic bacteria, etc.

57. Peptic Digest Agar

Neutralized Peptic digest	1000 c.c.
Dibasic potassium phosphate	2 gms.
Agar shreds, 2 per cent	20 gms.

Adjust the reaction to P_H 7.0–7.2 and add agar shreds. Boil until dissolved. Bring up to volume with tap water. Check the reaction to P_H 7.0. Cool to 50–55° C. and add the beaten white of one egg per litre. Mix thoroughly. Stabilize at 15 pounds pressure for 45 minutes. Filter through cotton pads just wetted with boiling water. Check the reaction. Flask in measured amounts or distribute in test tubes. Sterilize in the autoclave at 15 pounds pressure for 30 minutes. Incubate at 37° C. for 24–48 hours.

58. Peptic Digest Broth

Peptic digest	1000 c.c.
Dibasic potassium phosphate	2 gms.

Adjust reaction to P_H 7.2. Stabilize in the autoclave for ½ hour at 15 pounds pressure. Filter through folded filter paper. Fill into tubes or in measured amounts in flasks. Autoclave at 15 pounds pressure for 15–30 minutes. Incubate 48 hours.

59. Peptone Medium for Methyl Red Test and for the Voges-Proskauer Reaction (Standard)

To 800 c.c. of distilled water add 5 grams of Proteose-Peptone, Difco, or Witte's Peptone (other peptones should not be substituted), 5 grams c. p. dextrose, and 5 grams dipotassium hydrogen phosphate (K_2HPO_4). A dilute solution of the K_2HPO_4 should give a distinct pink with phenolphthalein. Heat with occasional stirring over steam for 20 minutes. Filter through folded filter paper, cool to 20° C. and dilute to 1000 c.c. with distilled water. Distribute 10 c.c. portions in sterilized test tubes. Sterilize by the intermittent method for 20 minutes on 3 successive days.

60. Petroff's Medium

Glycerinated peptic digest broth	1 part
Egg white and yolk	2 parts
Gentian violet sufficient to make proportion 1:10,000.	

Glycerinated Peptic Digest Broth.—Sterile peptic digest broth P_H 7.0, add sufficient glycerin to make a 15 per cent concentration; sterilize in the Arnold sterilizer for 15 minutes at 100° C.

Eggs.—Sterilize the shells of the eggs by immersion for 10 minutes in 70 per cent alcohol or by pouring hot water upon them. Break the eggs into a sterile breaker and after mixing the eggs well, filter through sterile gauze. Add one part by volume of the peptic digest broth.

Gentian violet.—Add sufficient gentian violet in 1 per cent alcohol to make a dilution of 1:10,000. Tube aseptically from 3–4 c.c. in each sterile test tube and inspissate at a temperature of 75–85° C. for 3 consecutive days. (The temperature should be brought up slowly to avoid bubbling.) Incubate 48 hours.

Use: For the isolation and cultivation of the tubercle bacillus.

61. Plain Potato Medium

After cutting potatoes into slices of the proper size, wash in running tap water over-night. Place a small piece of absorbent cotton in the bottom of a test tube. Place a slice of potato on the cotton and add distilled water until the potato is about ¼ immersed. (If the potato is very acid, a little Na_2CO_3 should be added to the distilled water. The reaction can be measured by adding a drop of brom-thymol blue to the surface of the potato slab.) Sterilize in the Arnold sterilizer for 20 minutes at 100° C. on 3 successive days.

62. Russell's Double Sugar Agar

Liebig's extract agar (Standard)	100.0 c.c.
Lactose (sterile, 25 per cent solution)	4.0 c.c.
Glucose (sterile, 25 per cent solution)	0.4 c.c.
Andrade's indicator	1.0 c.c.

Tube aseptically and heat in the Arnold at 100° C. for 20 minutes. Slant with a deep butt. Incubate 24 hours to test sterility.

63. Sabouraud's Glucose or Maltose Agar

Water (distilled)	1000 c.c.
Peptone	10 gms.
Crude glucose or maltose	40 gms.
Boil up, then add	
Agar (finely shredded)	18 gms.

Put into litre flasks. Autoclave for 5 minutes at 15 pounds pressure. Filter through cotton. Tube or flask in 200 c.c. amounts. Sterilize in the autoclave for 15 minutes at 15 pounds pressure.

Use: For the cultivation of fungi, yeasts and molds.

64. Saccharose Broth

To 1000 c.c. of stock veal infusion broth or peptic digest broth, P_H 7.2–7.4, add 10 grams (1 per cent) of saccharose, heat to dissolve and tube or fill in Smith fermentation tubes. Autoclave for 15 minutes at 15 pounds pressure.

Use: For stool analysis.

65. Semi-solid Agar

Prepare in the manner described under hormone agar but add 0.5 per cent agar instead of 1.5 per cent. Beef testicle may be substituted for the lean beef or beef heart.

Use: For the preservation of gonococcus.

66. Semi-solid Carbohydrate Agar

Prepare in the manner described under Liebig's Meat Extract carbohydrate broth (Standard) formula 43, but add 0.5 per cent agar shreds.

Use: For carbohydrate fermentation test.

67. Sodium Oleate Blood Agar (Avery)

To 94 c.c. of hot (90° C.) hormone agar (P_H 7.0–7.4) add 5 c.c. of a 2 per cent neutral sodium oleate solution (Kahlbaum's) and 1 c.c. of a sterile red blood cell suspension. The red blood cells are prepared by centrifugalizing either sterile rabbit's or sheep's blood, decanting the clean serum and making up to the original volume with veal infusion or Liebig's broth.

Use: For the isolation of *B. influenzae.*

68. Starch Agar (Standard)

Add 0.2 per cent of water soluble starch to formula 40.

69. Storage of Serum

Ox-blood is obtained from the abattoir in large sterile buckets. Sterile bricks are placed on the clot to help separate the serum. The serum is syphoned off aseptically 24 and 48 hours after collection. Fill in sterile bottles provided with sterile rubber stoppers or paraffinated cork stoppers. Add 1–2 per cent chloroform and store in ice chest. When ready for use decant the clear serum and evacuate the chloroform. The serum is used for the preparation of Loeffler's serum. The clot is used in the preparation of tryptic blood clot digest which serves as a very inexpensive and efficient substitute for veal infusion media.

70. Sugar-free Veal Agar

Sugar-free veal infusion broth	1000 c.c.
Agar	20 gms.

Melt and adjust to P_H 7.2. Cool to 55° C. and add the beaten white of one egg per litre. Stabilize ½ hour at 15 pounds pressure in the autoclave. Filter through cotton pads just wetted with hot water. Check the reaction to P_H 7.0–7.4. Flask in litre flasks or smaller amounts and autoclave at 15 pounds pressure for 30 minutes.

71. Sugar-Free Veal Broth

To sterile flasks of double strength veal infusion is added a fluid culture of *B. saccharolyte.* Incubate over-night. Boil in steam kettle 10 minutes and add equal volume of tap water. Add

Peptone (Difco)	1.0 per cent
NaCl	0.5 per cent

Adjust reaction to P_H 7.2–7.4. Bottle. Stabilize in autoclave ½ hour at 15 pounds pressure. Filter through paper, check the reaction, distribute in sterile flasks or test tubes and sterilize for 15–30 minutes at 15 pounds pressure.

72. Testicular Agar

Distilled water	500 c.c.
Agar	20 gms.
Dibasic potassium phosphate	3 gms.

Adjust reaction to P_H 7.2. Autoclave ½ hour. Mix aseptically with testicular infusion 500 c.c. Slant and incubate 24 hours.

Use: For the cultivation of *Micrococcus gonorrhoeae.*

73. Testicular Infusion

Minced beef testicles	500 gms.
Distilled water	500 c.c.

Heat until the albumen is coagulated. Strain through cheese cloth. Adjust reaction to P_H 7.2–7.4. Sterilize at 15 pounds pressure for 15 minutes.

74. Toxin Medium

Minced veal	1000 gms.
Distilled water	1000 c.c.

Infuse the mixture over-night in ice box. Boil for 15 minutes and strain through gauze. Add 0.5 per cent sodium chloride.

Shake into the boiling infusion 2 per cent peptone (Berna). (Stir constantly to avoid clumping.) Readjust reaction to P_H 7.6. Measure into bottles and cover with 1 cm. layer of vaseline. Autoclave for ½ hour at 15 pounds pressure. Add 2 per cent sterile glucose aseptically, and a layer (0.5–1 cm.) of sterile vaseline. Heat in the Arnold 30 minutes. Incubate 48 hours at 37° C.

Use: For the production of *Cl. botulinum* or *tetanus* toxin.

75. Veal Infusion
(double strength)

Chopped lean veal	1000 gms.
Water	1000 gms.

Boil ½ hour. Filter through cotton. Bottle while hot. Autoclave at 15 pounds pressure for 30 minutes.

Store for stock media. Before being used the fat is removed by filtering the infusion through several thicknesses of fine mesh gauze and an equal volume of tap water is added.

Use: As a base for broth, agar, etc.

76. Veal Infusion Agar

Veal infusion (double strength)	500 c.c.
Tap water	500 c.c.
Sodium chloride	5 gms.
Peptone (Difco)	10 gms.
Agar shreds	20 gms.

Dissolve the ingredients and adjust the reaction as desired P_H 7.0–7.6. Boil for 5–10 minutes. Make up to volume. Check the reaction. Cool to 55° C. and add the beaten white of one egg. Mix thoroughly. Stabilize in the autoclave at 15 pounds pressure for 45 minutes. Filter through a layer of gauze and cotton just wetted with boiling water. Check the reaction. Distribute in flasks or test tubes and sterilize at 15 pounds pressure for 30 minutes. Incubate at 37° C. for 24 hours.

77. Veal Infusion Broth

Double strength veal infusion	500 c.c.
Tap water	500 c.c.
Sodium chloride	5 gms.
Peptone (Difco)	10 gms.
Dibasic potassium phosphate	2 gms.

Dissolve ingredients by boiling 10 minutes. Adjust the reaction to P_H 7.6. Boil for 5 minutes. Check the reaction to P_H 7.6. Stabilize in the autoclave at 15 pounds pressure for 30 minutes. Filter through paper. Distribute in flasks or nursing bottles in measured amounts. Autoclave at 15 pounds pressure for 30 minutes. Incubate for 24–48 hours at 37° C.

78. Van Ermengen's Medium (modified)

Double strength veal infusion	1000 c.c.
Sodium chloride	5 gms.
Dibasic potassium phosphate	5 gms.
Peptone, Witte's	10 gms.

Dissolve by boiling. Adjust the reaction to P_H 7.4–8.0. Boil 10 minutes and check the reaction P_H 7.4–8.0. Fill into litre bottles. Autoclave at 15 pounds pressure for 30 minutes. Filter through paper. Bottle in 200 c.c. amounts. Autoclave ½ hour at 15 pounds pressure. Add aseptically a 2 per cent glucose (8 c.c. of a 50 per cent glucose solution) and a layer (0.5–1 cm.) of sterile vaseline. Heat in the Arnold sterilizer for 15 minutes at 100° C. Incubate at 37° C. for 48 hours.

Use: For the production of *Cl. botulinum* toxin.

THERMOMETRIC EQUIVALENTS

Cent.	*Fahr.*	*Cent.*	*Fahr.*
110	230	40.5	104.9
100	212	40.0	104.0
95	203	39.5	103.1
90	194	39.0	102.2
85	185	38.5	101.3
80	176	38.0	100.4
75	167	37.5	99.5
70	158	37.0	98.6
65	149	36.5	97.7
60	140	36.0	96.8
55	131	35.5	95.9
50	122	35.0	95.0
45	113	34.0	93.2
44	111.2	33.0	91.4
43	109.4	32.0	89.6
42	107.6	31.0	87.8
41	105.8	30.0	86.0

RELATION OF ENGLISH AND METRIC SYSTEMS

1 grain	64.8 m.g.
1 ounce	28.3 gms.
1 pound	453.6 gms.
1 gram	15.432 grains
1 kilo	2 lbs., 3 ozs.
1 minim	0.059 c.c.
1 fl. dram	3.5 c.c.
1 fl. ounce	28.39 c.c.
1 pint	567.9 c.c.
1 liter	35.2 fl. ozs.

INDEX

Absorption of agglutinins (exp.), 208.
Acetic toluidin blue stain, 384.
Acetyl-methyl-carbinol test, 399.
Acid agglutination (exp.), 200.
Acid-fast bacteria, 140–143.
  staining of, 385.
Acid mercuric bichloride, 383.
Acriflavine, dye as disinfectant, 101.
Actinomyces, 172.
Agar, *see* Media.
Agglutination
  absorption method (exp.), 208.
  acid (exp.), 200.
  Dreyer technic, 123.
  dysentery, 126.
  effect of salts on (exp.), 199.
  macroscopical, 123, 125.
  meningococci, 166.
  microscopical, 118, 124.
  pneumococcus, 156, 162.
  production of agglutinins (exp.), 202.
    for erythrocytes (exp.), 239.
  scarlet fever diagnosis by (exp.), 237.
  slide, 119, 166.
  staphylococcus (exp.), 230.
  streptococcus (exp.), 235.
Agglutinins in milk (exp.), 217.
Air, bacterial examination of, 86.
Alcoholic preparations
  absolute, 379.
  acetone-alcohol, 379.
  acid alcohol, 379.
  ether-alcohol, 380.
Allergy (exp.), 250–255.
Aleuronat, preparation for obtaining leucocytes, 218, 220.
Alexin, *see* complement.
Alkaline egg medium, 403.
Amboceptor, production of, 244.
Amoeba, *see* Entamoeba.
Ammonia
  production by bacteria, 46.
  test in bacterial cultures, 398.
Ammonium oxalate solution for blood culture, 380.
Ammonium oxalate gentian violet (Gram stain), 380.
Anaerobes, 175.
  Cl. botulinum, 179.
    experimental work, 260.
    State Hygienic Laboratory diagnosis of, 299.
  Cl. tetani, 176.
    experimental work, 258.
  Cl. welchii, 136, 175.
  Buchner method of cultivation, 38.
  Burri tube for cultivation, 39.
  isolation of, 38, 175.
  media for, 404, 410, 417, 418, 419, 421.
  M'Intosh and Fields jar, 39.
  purification of, 176, 178.
  Smith-Noguchi method, 38.
  toxicity tests, 179.
  Wright's method, 39.
Anaphylaxis (exp.), 250–255.
Anatoxin (exp.), 226.
Andrade's indicator, 380.
Anilin water for anilin gentian violet, 380.
Animal, experimental
  autopsy procedure, 68.
  bleeding of, 67.
  feeding of, 52, 56.
  hematology, 64.
  inoculation methods, 59.
  observation of, 64.
  spontaneous infections in, 52–56, 61.
  temperature and pulse of, 65.
Anthrax, 109.
  diagnosis of infection by coctoprecipitins (exp.), 215.
  State Hygienic Laboratory diagnosis, 299.
    specimens required for, 322.
  vaccination, cuti-immunity (exp.), 196.
Antianaphylaxis (exp.), 253.
Antibody, reticulo-endothelial beds in production of (exp.), 220.
Antibody production (exp.), 202.
Antiformin for concentrating tubercle bacilli, 141.
Antigen, preparation of, 121, 208.
Antistaphylolysin (exp.), 232.
Antisera, production of (exp.), 202.
Antitoxins for
  Cl. botulinum, 260.
  C. diphtheriae, 229.
  Cl. tetani, 258.
Arnold sterilizer, 15, 30.
Aronson's media, 403.
Arthus phenomena (exp.), 252.
Aspergillus, 89.
Autoclave sterilization, 15, 30.
Autogenous vaccine, preparation of, 289.
Autopsy of experimental animals, 68, 109.
Avery's broth, 404.

Bacillus
abortus, 133.
acid-fast, staining of, 140, 385.
acidophilus, 116, 210.
anthracis, 109.
State Hygienic Laboratory diagnosis of, 299.
spore bearer related to, 113.
avisepticus, 134.
bifidus, 116.
coli, 90, 120.
in water, 128.
dysenteriae, 126.
State Hygenic Laboratory diagnosis of, 303.
experimental work on toxins, 216.
fermentation reactions, opp. 120.
enteriditis, opp. 120, 207.
fluorescens, 90.
Friedlander's bacillus, 163, opp. 120.
influenzae, 169.
leprae, 143.
State Hygienic Laboratory diagnosis of, 306.
mallei, 172.
melitensis, 126, opp. 120.
mucosus capsulatus, 163.
murisepticum, 134.
of Bordet-Gengou, 170.
paratyphosus (A and B), 116, opp. 120.
State Hygenic Laboratory diagnosis of, 320.
pertussis, 170.
pestis 135.
State Hygenic Laboratory diagnosis of 308.
pneumoniae, 155.
State Hygienic Laboratory diagnosis of, 308.
proteus, 116, opp. 120.
pyocyaneus, 90.
smegmatis, 143.
subtilis, 84, 112.
swine erysipelas, 136.
tuberculosis, 140.
State Hygienic Laboratory diagnosis of, 319.
tularensis, 135.
typhosus, 116.
State Hygienic Laboratory diagnosis of, 320.
stool enrichment methods, 117, 120.
Bacteria
biochemical characteristics, 43.
classification of, 40.
counting of, 129, 137.
complement fixation of (exp.), 247–249.
cultivation of, 23, 175.
disinfection, applied to, 93.
effect of opsonins on (exp.), 220.
ferments of, 43, 91.
in air, 86.
in blood, 287.
in buccal cavity, 144, 153, 164, 225, 275, 301.
in food, 261, 299, 325.
in milk and ice cream, 136.
in soil, 175, 261.
in urine, 140, 277.
in water, 128.
resistance to drying, 94.
resistance to heat, 60° C., 95.
resistance to heat, 100° C., 96.
Bacteriolysis
Pfeiffer's phenomena (exp.), 197.
of normal sera (exp.), 189.
Bacteriophage (exp.), 267.
Bacterioprecipitins (exp.), 216.
Bacteriotropins (exp.), 222.
Barber single cell technic, 37.
Benian's Congo red negative stain, 386.
Bismark brown, 380.
Biochemical tests, 40, 397.
Biuret reaction, 420.
Blood
agar, 404, 410, 443.
preparation of, 144.
collection of, 67, 122, 284.
counts of experimental animals, 64.
culture, technic, 67, 286, 320.
flukes, 337, 360.
grouping (exp.), 239.
parasites in, 180, 306, 334, 352, 368.
serological examination of, 67, 121, 262, 310.
serum storage, *see* Media.
stain, identification method (exp.), 215.
thick film, preparation, 65, 335.
Bordet-Gengou bacillus, 170.
medium, 405.
Bordet-Gengou phenomena, *see* Complement-fixation.
Botulism
protective power of antitoxin (exp.), 260.
soils and foods (exp.), 261.
State Hygienic Laboratory diagnosis of, 299.
specimens required for, 323.
typing of toxin, 179.
Bovine tuberculosis, 143, 223.
Breed count of milk, 138.
Brilliant green as disinfectant, 104.
enrichment of typhoid paratyphoid, 120.
typhoid culture media, 406.
Brownian movement, 84.
Brucella group of organisms, 133.
Buccal cavity, organisms in (exp.), 225.
Buchner's anaerobic technic, 38.
Buffer index of media, 31.
Burke's spore stain, 394.

Burri
anaerobic technic, 39.
negative staining method, 386.
single cell technic for purification of bacterial culture (exp.), 190.

Capillary pipettes, 13.
Capsule, staining of, 387.
Carbohydrate media, *see* Media.
Carbolfuchsin stain, 381.
Carbolic acid disinfectant, 105.
solution, 380.
Cestodes (tape-worm), 363.
Chemotropism (exp.), 262.
Chemotherapy (exp.), 263.
Cholera bacillus, 127.
cholerá-red reaction, 127, 397.
Dieudonne's media for, 408.
specimens required for diagnosis, 323.
Chromogenesis, 47, 91.
Ciliates, intestinal, of man, 348.
Citrate solution, 384.
Classification of unknown organisms, 40.
Cleaning glassware, 11.
Cleaning slides, 12.
Cleaning solution, 11.
Clinical diagnosis of specimens, 271.
bladder, 277.
blood, 284.
central nervous system, 288.
duodenum, 279.
eye, ear, mouth and sinuses, 275.
feces, 280.
intestinal, 279.
lung juice, 276.
pericardium, 286.
peritoneum, 281.
prostate, 278.
respiratory tract and pleura, 275.
skin and subcutis, 282.
sputum, 276.
stomach, 279.
surgical cases, 282.
tooth cultures, 282.
urethra and vagina, 277.
vaccine preparation, 289.
Coagglutination, 208.
Cobra venom hemolysins, *see* Zoötoxins.
Coccidioides, 174.
Coccidia, 351.
Coccidiosis in rabbits, 174.
Coctoprecipitins (exp.), 215.
Colon bacillus, 90, 117.
differentiation by sugar fermentation, opp. 120.
in spinal fluids, 169.
in water, 128.
Colony fishing, 36.
Colostrum, agglutinins in (exp.), 217.
Complement
anticomplementary property of egg albumen (exp.), 248.
fixation, bacterial (exp.), 247.
fixation, in tuberculosis (exp.), 249.
fixation, in Wassermann, 263, 310.
properties and titration (exp.), 243.
Conversion table of weights, measures, and temperatures, 426.
Copper sulfate solution for spore stain, 381.
Corrosive sublimate fixation, 76.
Count of vaccines, 291.
Cutaneous tests (exp.),
allergic, 252.
Dick test, 237.
fluid extract for diagnosis, 255.
Kellogg test, 288.
Schick test, 227.
von Pirquet, 224.
Culture media, *see* Media.
Cuti-infection and cuti-immunity in Anthrax (exp.), 196.
Cyanochin, Eisenberg's negative stain, 22, 386.

Dark field illumination, 17, 20.
Dengue fever, specimens, 323.
Desensitization and Arthus phenomena (exp.), 253.
Dick test for scarlet fever (exp.), 237.
Dieudonné's medium, 408.
Diphtheria bacillus, 144.
Anatoxin (exp.), 226.
antitoxin standardization (exp.), 229.
complement-fixation test (exp.), 248.
diagnosis of, 144.
State Hygenic Laboratory technic, 301.
specimens required, 324.
differentiation by sugar fermentation, 147.
immunity of rats to (exp.), 226.
Kellogg test (exp.), 228.
Schick reaction (exp.), 227.
staining of, 301, 384, 393.
toxin production (exp.), 225.
virulence determination, 145, 148, 228.
Diphtheroids, 149.
Diplococcus
gonorrhoeae, 172.
meningitidis, 164.
pneumoniae, 155.
Disinfection
bichloride of mercury, 99.
brilliant green, 104.
carbolic acid, 105.
clothing, 96.
excreta, 108.
flavine dyes, 101.
formalin and chloroform, 97.
Gentian violet, 103.
hands, 107.
light, 106.
liquor cresolis, 98.
mercurochrome, 103.

phenol coefficient, determination of, 105.
preparation of cultures for, 94.
tricresol, 289.
water, 108.
Dorsett's egg medium, 407.
Dreyer's agglutination technic, 123.
Drying agar plates, 117.
Duodenal contents, 279.
Dunham's peptone, 408.
Dysentery amoebic, *see* Entamoeba.
Dysentery bacillus, 126.
differentiation by sugar fermentation, opp. 120.
State Hygienic Laboratory diagnosis of, 303.
specimens required, 324.
immunization with (exp.), 207.
toxins of (exp.), 216.

Ear, clinical specimens from, 275.
Ehrlich's indol test, 397.
Eisenberg's cyanochin relief stain, 386.
Empyema in rabbit (exp.), 233.
Encephalitis, 324.
Endo medium, 408.
Endospores, presence of, 42.
Entamoeba, 338.
Enteriditis bacillus (Gaertner), 325.
fermentation reactions of, opp. 120.
immunization of animals (exp.), 207.
Enzymes, 43, 91.
Eosin-methylene blue medium, 409.
Erysipelas, streptococcus of, 151.
specimens for diagnosis, 324.
Eye, clinical specimens from, 275.

Feces
collection of, 280.
culturing technic, 117, 120, 126, 127, 303, 321.
parasites in, 336, 338, 347, 348, 351, 356, 363, 368.
routine clinical examination, 116, 210.
tubercle bacilli in, 140.
Fever (exp.), 191.
Film
moist preparation of, 20.
stained preparation of, 21.
Filter funnels, 289.
Filter pads for media, 30.
Filterable virus diseases, 181.
Flagella stains, 388.
Flagellates, intestinal, in man, 347.
Flukes, 356.
specimens for diagnosis, 324.
Focal infection in rabbit (exp.), 235.
Food poisoning, 179, 325.
Formaldehyde as disinfectant, 97.
preservative, 121, 208.
tissue fixation by, 75.
Fowls, 56.
Fixation of tissues, 75.
Friedlander's bacillus, 163.
Fuchsin, 381.
Fuller titration of media, 26.
Fungi
cultivation of, 90.
pathogenic, 173.
Sabouraud's agar for, 422.
saprophytic molds, 89.
yeasts, 88.
Fusiform bacilli in Vincent's angina, 180.

Gabbett's stain, 319.
Gaertner bacillus, *see* B. enteriditis.
Gastric contents, 279.
Gelatin medium, *see* Media.
liquefaction, 43, 91.
Gentian violet
ammonium oxalate, 380.
anilin water, 381.
aqueous, 381.
carbol, 381.
in disinfection, 103.
selective action in media, 105.
Sterling's, 384.
Giardia intestinalis, *see* Flagellates.
Giemsa stain, 390.
Glander's bacillus, *see* B. mallei, 172.
specimens required, 325.
Glassware
cleaning and preparation of, 11.
sterilization of, 14.
Glossary of terms used in descriptive chart, 49.
Gnezda indol test, 397.
Gonococcus, 172.
State Hygenic Laboratory diagnosis of, 304.
specimens required, 326.
Gorè's indol test, 398.
Gram's iodine solution (Lugol's), 381.
Gram stain
Hucker and Conn, modification, 392.
Kopeloff and Beerman, modification, 392.
mechanism of (exp.), 188.
Papperheimer's stain for organisms in tissue, 77.
Greenspoon's medium, 412.
Greenthal's diphtheria stain, 393.
Growth, optimum PH for (exp.), 187.
table of, 29.

Hemophilic organisms, 169.
Hanging block, for fungi cultures, 173.
Hanging drop, 19, 84.
Hay fever (exp.), 254.
Heat in destruction of bacteria, 14, 30, 35, 95.
Hemoglobinophilic bacteria, 169.
Hemolysins (exp.),
heterophylic antigen for, 244.
in vivo, 245.

normal, of dog or goat serum, 242.
production of, 244.
staphylococcus, 231.
streptococcus, 233.
titration of, 244, 312.
Hemoprecipitans (exp.), 215.
Hemopsonins (exp.), 222.
Hemorrhagic septicemia group
B. avisepticus, 134.
B. pestis, 135, 308, 327.
B. murisepticum, 134.
Hemosporidia, *see* Malarial parasites.
Herpes, production of (exp.), 265.
Heterophylic antigens (exp.), 244.
Hiss, capsule stain, 387.
Hiss, serum water, 407.
Histological technic, 75.
Hog, cholera bacillus of, opp. 120.
Hookworm, diagnosis of, 304, 368.
specimens for diagnosis, 326.
Hot air sterilization, 14.
Hucker and Conn, modified Gram stain, 392.
Hydrogen-ion, determination in media, 27.
optimum growth range, 29.
Hydrogen sulfide determination in cultures, 399.
Hay fever, 254.

Immune sera, production of
antibacterial, 202.
hemolytic, 244.
reticulo-endothial beds in (exp.), 220.
Impression preparations, 111.
Instruments, care of, 16, 60.
Indicators
hydrogen-ion, 27.
brom cresol purple, 406.
Andrade's, 380.
Indol
test, 397.
production of, 47.
Infections, in laboratory animals, 52.
Inflammation (exp.), 191.
Influenza bacillus, 169.
material for diagnosis, 326.
spinal fluid, 169.
Infusion, organic, 84.
Instruments, care of, 16.
Intestinal parasites, 304, 338, 347, 348, 351, 356, 363, 368.
Inoculation of
animals, 59.
media, 38.
Intestinal flora (exp.), 116, 210.
Involution forms of bacteria (exp.), 21, 187.
Iodine solution, Lugol's, 381.
Iodine, tincture of, 384.
Iso-agglutinins and hemolysins (exp.), 239.
Isolation of bacteria in pure culture, 33.
animal inoculation, 37.
Barber technic, 37.
Burri technic (exp.), 190.
colony fishing, 32, 36.
pour plates, 34, 154.
streak plates, 35, 97.

Jansky's blood grouping, 239.

Kahn test, 262.
Kala-azar, *see* Leishmaniae.
Kellogg test for diphtheria immunity (exp.), 288.
Klebs-Loeffler bacillus, *see* Diphtheria, bacillus of.
Kofoid-Barber method of fecal examination for parasites, 336.
Kolmer's modification of Wassermann test, 262, 310.
Konrich's acid-fast stain, 385.
Kopeloff and Beerman, modified Gram stain, 392.

Laboratory consultation, form for, 274.
Lacto-phenol, 382.
Leishmaniae, 341.
Leprosy, bacillus of, 143.
State Hygienic Laboratory diagnosis of, 306.
specimens required, 326.
Leucocidin (exp.), 218.
Leucocytosis (exp.), 217.
Light, destruction of bacteria by, 106.
Liebig's meat extract media, *see* Media.
Liquor cresolis compositus, 382.
Liver flukes, 358.
Lobar pneumonia in rabbit (exp.), 238.
Loeffler's medium for diphtheria, 417.
Loeffler's methylene blue, 382.
Lugol's solution, 381.
Luminescent bacteria (exp.), 188.
Lung flukes, 359.
Lung juice, collection of, 276.

Macrophages (exp.), 218.
Malaria parasites, 352.
diagnosis of, 306, 354.
specimens for diagnosis, 326.
Malignant edema, *see* Vibrion septique.
Malta fever bacillus, *see* B. melitensis.
Martin's broth, 420.
Measles, specimens for diagnosis, 325.
Media, essentials in preparation and sterilization, 23, 92.
adjustment of reaction, 26.
alkaline egg medium (No. 1), 403.
Aronson's medium (No. 2), 403.
ascitic fluid agar (No. 3), 403.
Avery's broth (artificial mouse), (No. 4), 404.
beef heart medium (No. 5), 404.
blood agar (No. 6), 404.
chocolate (No. 7), 404.

glucose peptic beef heart (No. 26), 410.
laked (No. 8), 404.
sodium oleate (No. 67), 423.
blood broth (cooked) (No. 9), 405.
Bordet-Gengou for B. pertussis (No. 10), 405.
brain medium (No. 11), 405.
brain suspension (No. 12), 405.
brilliant green-eosin agar (No. 13), 406.
brom cresol purple milk (No. 14), 406.
buffer index of, 31.
carbohydrate media (No. 15), 407.
colored plugs for, 24.
Hiss serum water (No. 16), 407.
sterilization of, 24, 30.
clearing of, 29.
Dorsett's egg medium (No. 17), 407.
Dieudonné's media for cholera bacilli (No. 18), 408.
drying of agar plates, 117.
Dunham's peptone solution (Indol test), (No. 19), 408.
Endo's agar (No. 20), 408.
eosin-methylene blue agar (No. 21), 303, 409.
gelatin (No. 22), 409.
glucose beef juice, broth (No. 23), 409.
glucose brain, broth (No. 24), 410.
glucose peptic beef heart (No. 25), 410.
glucose peptic beef heart, blood agar (No. 26), 410.
glucose peptic beef heart, broth (No. 27), 411.
glucose peptone solutions (standard), (No. 28), 411.
glucose veal broth, for blood cultures (No. 29), 412.
glycerin peptic digest, agar (No. 30), 412.
glycerin peptic digest, broth (No. 31), 412.
glycerin veal, agar (No. 32), 412.
glycerin infusion, broth (No. 33), 412.
Greenspoon's medium (No. 34), 412.
Hiss serum water (No. 16), 407.
hydrogen-ion determination of, 27, 29.
inoculation of, 31.
lactose beef infusion, broth (No. 35), 412.
lactose peptone bile (No. 36), 413.
lead acetate, agar (No. 37), 413.
Liebig's meat extract
agar (for milk counts), (No. 38), 414.
agar (No. 39), 414.
agar (standard), (No. 40), 415.
broth (No. 41), 415.
nutrient broth (No. 42), 415.
carbohydrate broth (standard), (No. 43), 415.
gelatin (standard), (No. 44), 416.
liver infusion (stock), (No. 45), 416.
liver infusion, agar (anaerobic shake cultures), (No. 46), 417.
liver infusion, broth (No. 47), 417.
Loeffler's serum medium, for diphtheria (No. 48), 417.
Nicolle's medium (No. 49), 418.
nitrate, agar (No. 50), 418.
nitrate, broth (No. 51), 418.
nitrate, broth (standard), (No. 52), 418.
ox-gall medium (No. 43), 418.
pancreatic digest of casein (No. 54), 418.
pea peptic gelatin (No. 55), 419.
peptic digest (Martin's broth), (No. 56), 420.
peptic digest, agar (No. 57), 420.
peptic digest, broth (No. 58), 421.
peptone medium, for methyl red and Voges-Proskauer (standard), (No. 59), 421.
Petroff's medium for tubercle bacilli (No. 60), 421.
potato (No. 61), 422.
Russell's double sugar, agar (colon-typhoid), (No. 62), 422.
Sabouraud's agar for fungi (No. 63), 422.
saccharose, broth (No. 64), 422.
semi-solid, agar (No. 65), 422.
semi-solid, carbohydrate agar (No. 66), 423.
sodium oleate blood-agar (Avery's), (No. 67), 423.
stabilization of, 29.
standardization of, 25.
starch agar (standard), (No. 68), 47, 423.
sterilization of, 30.
storage of serum (No. 69), 423.
sugar-free veal, agar (No. 70), 423.
sugar-free veal, broth (No. 71), 424.
testicular, agar (No. 72), 424.
testicular infusion (No. 73), 424.
titration of, 26.
toxin medium (No. 74), 424.
veal infusion (double strength), (No. 75), 425.
veal infusion, agar (No. 76), 425.
veal infusion, broth (No. 77), 425.
van Ermengem's medium (modified), (No. 78), 426.
Mercuric bichloride, 383.
acetic acid, 76.
acid, 379.
Methyl red
stain, 382.
test, 399.
Methyl violet, carbol, 382.
Methylene blue, Loeffler's, 382.

Meningococcus, 164.
agglutination of, 166.
carriers, 167.
cultivation of, 164.
media for, 164.
spinal fluid, examination of, 108, opp. 288.
State Hygienic Laboratory diagnosis of, 306.
specimens for, 323.
Mice, use and care of, 54.
Micrococcus
catarrhalis, 165, 166, opp. 276.
crassus, 165, 166.
flavus, 166.
intracellularis meningitidis, 164.
melitensis, 133.
pharyngis siccus, 165.
tetragenus, 149, 153.
Microphages (exp.), 219.
Microscope, use of, 17.
Milk bacteriology, 136.
agglutinins in (exp.), 217.
Breed method of counting bacteria, 138.
cultural method of counting bacteria, 137.
reductase test, 136.
test for B. welchii, 136.
Moeller's spore stain, 394.
Monilia, 173.
Motility, definition of, 42, 84.
Molds, *see* Fungi.
Mouth, bacteria in, 153, 225, 275.
clinical specimens from, 275.
Mucor mucedo, 89.
Muir's capsule stain, 387.
Mumps, 326.
Mutation of B. coli (exp.), 190.

Nares, external, 275.
Naso-pharynx, specimens from, 167.
Needles, use and care of, 60.
Negative staining, 22.
Negri bodies, diagnosis of rabies, 182, 309.
Nematoda, 368.
Nervous system, collection of specimens, 288.
Nephrotoxin (exp.), 256.
Neutral red solution, 88, 383.
Neutral red counterstain, 383.
Nicolle's medium, 418.
Nitrite test, 45, 398.
Normal serum, bactericidal effect of (exp.), 189.

One cell culture (exp.), 190.
Opsonins (exp.), 220.
hemopsonins (exp.), 222.
Optimum PH of growth, 27, 29, 187.
Organic infusion, 84.
Organisms in Buccal cavity (exp.), 225.
Organotropism (exp.), 224.
Old tuberculin (exp.), 224.

Pappenheimer's acid-fast stain, 385.
Gram stain in tissues, 77.
Parasites, diagnosis and preservation of, 335.
Parasitropism (exp.), 263.
Paratyphoid A and B, *see* Typhoid.
Pasteur pipettes, 13.
Pasteurella group of organisms, 134.
Pencillium glaucum, 89.
Pericardial fluid, organisms in, 286.
Peritoneal fluid, collection of, 281.
Petroff's medium for isolating tubercle bacilli, 421.
Pfeiffer's bacillus, *see* B. influenzae.
Pfeiffer's phenomena (exp.), 197.
Phagocytosis (exp.), 218.
Phenol coefficient, 105.
Pirquet (von), skin reaction, *see* Tuberculosis.
Plague bacillus, 134.
State Hygienic Laboratory diagnosis of, 308.
specimens required, 327.
Plasmodium, *see* Malaria.
Plimmer and Paine, flagella stain, 388.
Pneumonia, type diagnosis of, 308.
specimens for diagnosis, 327.
Pneumococcus, 155.
Avery's rapid culture method, 161.
cultivation of, 155.
diagnosis of, by animal inoculation, 158.
lobar pneumonia in rabbit (exp.), 238.
meningitis, 169.
mouse inoculation, 160.
precipitin test, 157, 162.
protective value of antisera (exp.), 238.
spinal fluid diagnosis, 169.
sputum collection, 160.
sputum diagnosis, 159.
typing of, 156, 158.
urine, concentration of, 162.
Poliomyelitis, 327.
Pour plates, 34, 154.
Precipitation (exp.),
anthrax, diagnosis, 216.
blood cell identification, 215.
coctoprecipitins, 215.
Kahn test for syphilis, 262.
pneumococcus, 157.
production of, 214, 215.
thermoprecipitins, 216.
Problems, general directions for, 185.
Protective immunization of small animals (exp.), 201.
Protective value of antipneumococcus sera (exp.), 238.
Protein preparation, for skin testing (exp.), 255.
Protozoa, examination of feces for, 305.

Purification of cultures, 33.
animal inoculation, 37.
Churchman method, 103.
colony picking, 32.
dilution plates, 34, 154.
heating, 35.
shake, 31.
single cell, 37.
streak plate, 35, 97.
Pyemia due to staphylococcus (exp.), 230.
Pyocyanin, extraction of, 90.
Pyrogallic acid for obtaining anaerobiosis, 39.

Rabbit
empyema of (exp.), 233.
feeding of, 53.
injection of, 59.
spontaneous infections in, 53.
tuberculosis in (exp.), 141.
Rabies, 182.
State Hygienic Laboratory diagnosis of, 309.
specimens required, 327.
virus production (exp.), 265.
Rat, care and feeding, 55.
Reductase test in milk, 136.
Reportable diseases, 272.
Reticulo-endothelial beds in antibody production (exp.), 220.
Rickettsia bodies, 182.
Rocky Mountain spotted fever, diagnosis of, 310.
specimens required, 327.
Rules regarding care of animals, 58.
Russell's double sugar medium, 422.

Sabouraud's medium for fungi, 422.
Safety cap, preparation of, 14.
Saline physiological, 383, 384.
buffered, 383.
Salkowski, indol test, 397.
Salts, affect on agglutination (exp.), 199.
Scarlet fever
diagnosis of, 153.
by concentration of serum agglutinins, 237.
Dick test (exp.), 237.
production of causative toxin (exp.), 238.
specimens for diagnosis, 328.
streptococcus scarlatinae, biological reactions, 150.
Schick reaction (exp.), 227.
Schultz-Dale anaphylaxis technic (exp.), 252.
Septicemia (exp.), 232.
Shunk's flagella stain, 389.
Sinus, nasal, 275.
Skin and subcutis, collection of material from, 282.
Skin reactions, *see* Cutaneous tests.
Sleeping sickness, *see* Trypanosomae.
Slide agglutination, 119, 166.
Smallpox (exp.), 266.
specimens required for diagnosis, 328.
Smegma, bacillus, 143.
Smith fermentation tube, 195.
Smith-Noguchi method of isolating anaerobes, 38.
Snake venom, *see* Zoötoxins.
Sodium citrate solution, 384.
Soil, isolation of Cl. botulinum from (exp.), 261.
Specimens, general directions for examination of, *see* Clinical diagnosis.
Spinal fluid, examination of, 168, 288.
Spirochaetes, 180.
staining of, 386, 395.
Spore stains, 394.
Sprue, *see* Monilia.
Sputum
collection of material, 160, 276.
concentration by alcohol, 140.
microscopic examination of, 140, 160.
plating for vaccine, 161.
pneumococcus typing, 159.
tuberculosis diagnosis, 140, 319.
Staining methods, general directions, 21, 85.
Staining of bacteria
acid-fast, 385.
Gabbett's, 319.
Konrich's, 385.
Pappenheimer's, 386.
Ziehl-Neelsen, 385.
capsule stain
Hiss, 387.
Muir, 387.
differential stain, 22.
dead and living cells (exp.), 187.
Gram's staining method, 391.
principles underlying (exp.), 188.
flagella stain
Plimmer and Paine's, 388.
Shunk, 389.
Zettnow's, 390.
in tissue
Giemsa, 78.
Gram (Pappenheimer), 77.
for acid fast organisms, 78.
simple stain, 77.
negative staining, 22.
Benian's Congo red, 386.
Eisenberg's cyanochin, 386.
India ink, 386.
polychrome stains
Giemsa, 390.
Negri body, 182, 309.
Tilden's, for spirochaetes, 395.
Wright's, 395.
simple stains
carbol-fuchsin, 381.
Loeffler's methylene blue, 382.
safranin, 381.

tissue, 77.
toluidin blue, 384.
special polar body stains
Greenthal's stain, 393.
thionin, carbol, 385.
toluidin blue, 384.
Wayson's stain for B. pestis, 395.
spore stains
Burke's, 394.
Moeller's, 394.
vital stains, 20, 88.
methylene blue, 382.
neutral red, 383.
Staining solutions, saturated, 380.
Standardization of diphtheria antitoxin (exp.), 229.
Staphylococcus, 149.
agglutination of (exp.), 230.
disinfection of, 93.
fermentation reactions of, 149.
hemolysins (exp.), 231.
pyemia (exp.), 230.
Staphylolysin (exp.), 231.
antistaphylolysin (exp.), 232.
Starch, test for hydrolysis, 47.
Strauss test to diagnose glanders, 172.
Streptothrix or nocardia, 173.
Steam, destruction of bacteria by, 15, 30.
Sterling's gentian violet, 384.
Sterilization, 15, 30.
glassware, 14.
media, 30.
Stock cultures, 37.
Stools
amoeba in, 338.
cholera, 127.
culturing technic, 117, 120.
flora of, 116.
routine clinical examination of, 210.
Storage of sera, 423.
Streptococcus, 153.
agglutination (exp.), 235.
bile test, 153.
differentiation by hemolysis and fermentation, 149.
empyema (exp.), 233.
hemolysin (exp.), 233.
in the mouth and throat, 153, 225, 275.
meningitis, 169.
scarlatinae, *see* Scarlet fever.
surface tension, effect on growth (exp.), 234.
Streptolysin (exp.), 233.
Surface tension effect on streptococci (exp.), 234.
Surgical specimens, diagnosis of, 282.
Syphilis
Kahn precipitin test for (exp.), 262.
spirochaeta pallida, 180.
Wassermann reaction in, 262, 310.
Kolmer's technic, 310.
specimens required, 328.
Syringe, care of, 60.
Tapeworms, *see* Cestoda.
Tetanus, Cl.
anaerobic cultivation, 175.
antitelanolysin (exp.), 258.
antitoxin protective value in animals (exp.), 179, 260.
specimens required for diagnosis, 328.
toxin, natural immunity of hen against (exp.), 258.
media for production of, 424.
Thermoprecipitinogen (exp.), 216.
Thionin, carbol, 385.
Throat smears and examinations, 153, 225, 275.
collection of material, 275.
diphtheria, 144.
scarlet fever, 153.
Vincent's angina, 180.
Thrush, *see* Monilia.
Tilden's spirochaeta stain, 395.
Tincture of iodine, 384.
Tissue, histological examination of, 75.
Titration of
agglutinins, 123.
amboceptor, 312.
hemolysins, 244.
media, 26.
precipitins, 214.
Toluidin blue, polar stain, 384.
Tooth cultures, 282.
Toxin
Cl. botulinum in food and soil (exp.), 261.
diphtheria, production of (exp.), 225.
formalization of (exp.), 226.
streptococcus scarlatinae (exp.), 238.
tetanus (exp.), 258.
use in Dick test (exp.), 237.
use in Schick test (exp.), 227.
Trachoma, 328.
Trematoda, 356.
blood flukes, 360.
intestinal flukes, 357.
liver flukes, 358.
lung flukes, 359.
Treponema pallida, 180.
Trypanosomae, 344.
Tuberculin (exp.), 224.
Tuberculosis
bovine type differentiation, 143.
cultivation of bacilli on Petroff's medium, 141, 421.
diagnosis of, 319.
complement fixation (exp.), 249.
material required for, 328.
spinal fluids, 168.
sputum, urine, feces, 140.
in guinea pigs (exp.), 223.
in rabbits (exp.), 223.
isolation of, 141.
staining of bacilli, 78, 319, 385.

tuberculin, chemistry of (exp.), 224.
von Pirquet, skin reaction (exp.), 224.
Tularemia, bacillus of, 134.
Typhoid bacillus
agglutination by modified Dreyer technic, 123.
antigen preparation, 121.
blood culture, 286, 320.
diagnosis of organism by agglutination
macroscopic, 121, 123, 125.
microscopic, Widal, 124.
slide, 118.
complement fixation (exp.), 247.
differential media for, 406, 408, 409.
disinfection of contaminated material, 108.
fermentation reactions, opp. 120.
immunization of animals, 207.
isolation from feces, 117, 120.
by brilliant green enrichment, 129.
meningitis, 169.
microscopic agglutination, 166.
paratyphoid organisms, reactions of, opp. 120.
protection of small animals (exp.), 201.
State Hygienic Laboratory technic for diagnosis, 320.
specimens required, 329.
Widal reaction, 121.
collecting blood for, 122.
Dreyer technic for, 123.
microscopic, 124.
Typhus, diagnostic Weil-Felix reaction, 267, 322.
specimens required, 330.

Urine
collection of, 277.
pneumococcus in, 162.
tubercle bacilli in, 140.
Uro-genital specimens, collection of, 277.

Vaccine preparation, U. S. Public Health Standard (exp.), 192.
autogenous (clinical work), 289.
Vanillin test for indol, 397.
Venom hemolysins, *see* Zoötoxins.
Vibrio cholerae, 127.
Vibrion septique, 175.
Vincent's angina, 180.
Virulence tests for diphtheria, 145, 148, 228.
Virus, filterable (chickenpox), 323.
Voges-Proskauer reaction, 399.
van Ermengem medium, 426.
von Pirquet, tuberculin skin test (exp.), 224.

Wassermann reaction, 262, 310.
Water examination, 128.
Wayson's stain, for plague bacilli, 395.
Weil-Felix reaction, 267, 322.
Welch bacillus, test for, 136.
West tube, in taking naso-pharyngeal cultures, 167.
Whooping cough, *see* B. pertussis.
specimens for diagnosis, 330.
Willis method of fecal examination for parasites, 336.
Widal reaction, 121.
Worms, *see* Nematoda and Trematoda.
Wright's blood stain, 395.
anaerobic technic, 39.
capsules, 122.
opsonic index (exp.), 220.

Yeasts
pathogenic, 174.
saprophytic, 88.
spore formation in, 88.

Zettnow's flagella stain, 390.
Ziehl-Neelsen stain for tubercle abcilli, 385.
Zingher's modification of the Schick test (exp.), 227.
Zoötoxins (exp.), 255.

University of California Syllabus Series

No. 179

ERRATA

Page 28. Sixth line from bottom, $\frac{N}{10}$ should read '$\frac{N}{20}$'.

Page 67. Nineteenth line from top, sergin should read, 'sesqui'.

Page 116. Eighth line from botton should read, 'and by Gram's method'.

Page 123. Sixth row from bottom should read, '3.6 cc. of saline, making a 1:10 dilution'.

Page 124. Sixth row under tube 4, should read, '1:80'.

Page 129. Thirteenth line from bottom should read, 'have ready 11 sterile, etc.'.

Second line from bottom, change fifth to 'a' and ninth to 'tenth'.

Last line, tenth should read, 'eleventh'.

Page 140. Line 16 from top should read, 'uric acid crystals'.

Page 143. Line 12 from top should read, 'strained' instead of stained.

Page 150 and 151. Third column from left heading should read, 'reduction of nitrates to nitrites'.

Page 202. Last sentence should read, 'If the first series of injections on 3 successive days does not yield a sufficiently high titre, a second series is begun in the same manner 5 days after the last injection.'

Page 277. Sixth line from top should read, 'without interrupting the flow'.

Page 383. Ninth line from bottom should read, '($Na_2HPO_4 + 2H_2O$)'.

Between eighth and ninth lines insert, 'or'.